REPORTS

ON

PUBLIC HEALTH AND MEDICAL SUBJECTS.

No. 1.

THE COMPLEMENT FIXATION TEST IN SYPHILIS, COMMONLY KNOWN AS THE WASSERMANN TEST.

MINISTRY OF HEALTH.

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To the

Right Hon. CHRISTOPHER ADDISON,
M.D., F.R.C.S., M.P.,
Minister of Health.

SIR,

I BEG to submit a report prepared under the instruction of the Local Government Board on "The Complement Fixation Test in Syphilis," by Col. L. W. Harrison, Dr. Eastwood, Dr. F. Griffith and Dr. W. M. Scott. The report is of a technical character only, but it is hoped that it will prove useful both as a guide to medical men engaged in the diagnosis of syphilis and as a contribution to the advancement of research in this behalf.

I am, Sir,
Your obedient servant,
GEORGE NEWMAN,
Chief Medical Officer.

April 1920.

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I.—Introduction.

SIR,

I BEG to present to you a short account of the events which led up to the undertaking of the important researches described in these reports.

In 1914 a Sub-Committee of the Royal Society of Medicine was formed, under the chairmanship of Professor F. W. Andrewes, F.R.S., to consider the merits of the various methods of conducting the Wassermann test for syphilis which were then in vogue, and to make such recommendations as might seem advisable for improvement in its technique. During the period of its sittings Dr. (now Sir Arthur) Newsholme, K.C.B., Medical Officer of the Local Government Board, suggested to the chairman that, if further research were required to assist the Committee, a grant might be obtained from the Local Government Board. The best method of employing such a grant was considered at the next meeting of the Sub-Committee, and it was agreed that further research to clear up many points on which workers were in dispute was most desirable. The Military Hospital, Rochester Row, was chosen as the place for the research, on account of the control over patients which was possible in that institution, and it was eventually arranged with the War Office Authorities that I should carry out the research, reporting to the Committee from time to time. The war commenced less than a month after these arrangements had been completed and before a start had been made at Rochester Row. Early in 1917, after I had returned to Rochester Row, Sir Arthur Newsholme asked me if I could possibly take up the work with assistance from his own staff. I agreed to give any assistance I could from the resources in pathological material at Rochester Row and by placing my ideas at the disposal of the Local Government Board's bacteriologists—Drs. Eastwood, Griffith, and Scott. Sir Arthur Newsholme was good enough to suggest that the research should be under my direction, but I cannot be said to have filled that position, since other duties prevented me from keeping in such close touch with it as would be required of the director of an investigation into a subject of such great complexity. The research should therefore be regarded as having been conducted in all its main features by the present pathological staff of the Ministry of Health.

At the time of the commencement of this investigation the Special Committee on the Standardisation of Pathological Methods, appointed by the Medical Research Committee, had recently taken up the subject of the Wassermann test, and I had been co-opted for the purpose.

At the outset of their report (Medical Research Committee, Special Report Series, No. 14) this Committee voiced the desirability of a more or less uniform technique, but their report shows that they found it impossible to recommend, for universal adoption, any one standard method, on account of the differences of opinion between workers of repute as to the relative importance of various points in which their technique differed. Subsequently this Committee reviewed (Special Report Series, No. 21) the literature bearing on the concordance of results of the Wassermann test in different hands with what were believed to be the clinical facts and, themselves, conducted a special investigation, in which 104 samples of serum, from cases in which the clinical diagnosis was made independently, were each tested in three different laboratories. In two of the laboratories, which employed a closely similar technique, the result showed very close agreement, as well as concordance with the clinical findings, in a very high percentage of cases. At the same time, the report revealed again the desirability of a systematic research into the properties and proportions of the different ingredients employed in the Wassermann test and their behaviour under different physical conditions, in order to appraise at their true value those discrepancies in technique on which different workers are at present unable to come to an agreement.

The investigation, which has been in progress in the laboratory of the Local Government Board since July 1917, was designed to meet this want.

At the outset we agreed that it would be necessary to take each ingredient of the Wassermann test and work out its vagaries under all possible circumstances which might occur in the routine test. Having discovered the factors which make for variations in the behaviour of the several ingredients, it seemed likely that a method of reducing those variations to the lowest possible dimensions would be discovered, and the way become clear to the elaboration of a technique which would afford in all hands more consistently reliable results than were then being obtained. The results of the research, so far as it has proceeded, are embodied in the following reports by Drs. Griffith and Scott. The authors do not regard the conclusions as final, but it will be agreed by those who read the account of their experiments that they constitute an important advance in the technique of the Wassermann test. They make clear much that was formerly obscure, and they show the practical importance of certain findings by other workers which have not received the general acceptance they should. Variations in the behaviour of complement, and the absence of any relation between hæmolytic activity and fixability have been pointed out by many workers, notably by Browning and Kennaway. The present research shows that not only is there no constant relation between hæmolytic activity and fixability

but also that the behaviour of a complement towards antigen alone is no indication of what will happen when it is brought into contact with the serum-antigen complex. It is for this reason that, in the method of conducting the Wassermann test which is proposed in this paper, the amount of complement-containing serum is kept constant, a departure from the usual technique of the present day, in which the quantity of guinea-pig serum employed in the test is determined by the hæmolytic titre, both alone and in the presence of antigen.

Probably more discussion has raged over the properties of different "antigens" than over any other single constituent of the Wassermann test. Broadly, there are those who prefer plain extracts; those who fortify these with cholesterin; those who remove the acetone-soluble fraction of plain extract on the grounds of its containing undesirable compounds in variable amount; and those who use only the acetone-soluble fraction. Against the plain extract and the acetone-insoluble fraction is the contention that they fail to detect an important proportion of syphilitic sera. Against the addition of cholesterin to plain extract it is said that this is liable to result in false positives; although this may not be true of such "antigens" when employed with adequate controls and when fixation takes place at 37° C., it is certainly true when the method of prolonged fixation at ice-chest temperature is employed, as numerous tests carried out by Captains Wyler and Balthasar, R.A.M.C., at my suggestion, have shown. As a result of the present research, Doctors Griffith and Scott have concluded that one of the undesirables in the acetone-soluble fraction of a plain extract is its variable content of cholesterin; and that one of the main disadvantages of the acetone-insoluble fraction advocated by Noguchi is that, lacking cholesterin, it is not such a delicate reagent as it is desirable to employ in this test. The authors propose, therefore, to remove the natural cholesterin from the plain extract (along with undesirables which, like cholesterin, are soluble in acetone) and then to add cholesterin in the amount which is found by experiment to be appropriate. This seems to go far towards the production of a standard antigen which is both delicate and reliable. At the same time the different behaviour of "antigens" prepared from the same materials, which is disclosed by this research, indicates that comparative statistics on the subject should be based on tests conducted with the same "antigen." The desirability of issuing such an "antigen" from a central source is supported, also, on the ground that considerable labour is involved in its preparation and standardisation.

As to the method of preliminary fixation, there is much to recommend prolonged fixation in the ice-chest. Dean has shown that fixation at ice-chest temperature, though slower, is more complete than fixation at 37° C., which, in the time allowed, is apt to miss the slowly-fixing serum-antigen com-

plexes. There is no doubt that ice-chest fixation provides a larger percentage of positives in treated cases, but it has been somewhat discredited by the fact that it has given false positives. The present research indicates that these have been due to the use of crude extracts fortified by cholesterin; in a word, perhaps, to the use of too much cholesterin—the unknown contained in the crude extract plus that which is added artificially. The use of a carefully standardised extract, the cholesterin content of which is known, such an extract as is suggested here, should remove this disadvantage, whilst preserving the advantage of increased delicacy which the method of prolonged fixation in the cold affords. A slight disadvantage of the method proposed in this paper is that the tests cannot be completed in the same day. It is compensated by the advantage that the important process of reading results is conducted in the morning, when the light is good and the observer fresh.

It has been possible to mention only a few of the outstanding features of this research. Careful study of the report will suggest much to the reader that is worthy of his further investigation.

The study of the principles involved in the Wassermann test, which has been prepared by Dr. Eastwood, raises a number of points on which workers could profitably engage in scientific discussion and experiment. So far as I know, no critical review has been written on this section of the subject which is so complete as that presented here by Dr. Eastwood. To those who have hitherto been content to follow the directions for the Wassermann test which are given in the average text-book it may open out new lines of thought on this important subject.

Too many workers are content to follow blindly some technique which appeals to them as either simple, or as reputed to afford reliable results. They do not trouble themselves with any thought as to the principles of the test, and their work, though useful in its way, is merely mechanical. It is not in this manner that progress will be made. The best method extant is by no means perfect, in the sense of detecting the least trace of Wassermann substance, and of excluding every non-syphilitic serum, and it is only by patient research that we shall approach perfection. Much can be done in this direction by pathologists working for the Ministry under the V.D. Scheme. Though routine methods must necessarily be employed for the ordinary Wassermann tests of specimens from V.D. clinics, it is suggested that, working with the pathological material available, they could profitably engage in research designed to clear up points which are still obscure about the nature of the Wassermann test, some of which have been raised in these reports. Eventually, by the publication of such researches and conference between workers to decide

their relative importance to the technique, considerable progress should be made towards the desired goal.

Another point arises. Some time ago Dr. D'Este Emery suggested that samples of sera giving certain reactions should be circulated and used by different workers as standards by which to compare their results. The present research of Drs. Griffith and Scott confirms Dr. Emery's conclusion that sera retain their properties with regard to the Wassermann test sufficiently long for this purpose, and there is no doubt that, by the exchange and circulation of samples of human serum, a better idea would be obtained of the meanings of results recorded by one observer when interpreted in the terms employed by others. In the same connection it would be of great assistance to the continuation and co-ordination of research if workers would forward to the Ministry's laboratory samples of sera which, in their hands, have given doubtful reactions. Naturally, the samples should be from cases in which the clinical findings are, as far as practicable, indisputable.

The task of perfecting a serological test for syphilis is not one which a single worker can accomplish except by a stroke of genius or great good fortune. But the number of those now working with syphilitic sera is considerable; many of them are, moreover, in a position to correlate results with clinical findings, either by actual observation or by conference with clinicians. As mentioned above, if each of these would supplement his routine, mechanical work of conducting Wassermann tests by research on some one point which bears on the behaviour of syphilitic serum under different conditions the goal of a perfect test would quickly become nearer.

I am Sir,
Your obedient servant,
L. W. HARRISON.

Sir George Newman, K.C.B.,
Chief Medical Officer,
Ministry of Health.

April 1920.

II.—Technique of the Wassermann Reaction.

By Fred. Griffith, M.B., and W. M. Scott, M.D.

CONTENTS.

Introduction.

Though the Wassermann reaction possesses a high degree of reliability when due precautions are taken,* there are many questions of technique about which consensus of opinion has not yet been obtained. If these could be settled, the way would be prepared for more precise standardisation of technique, with the twofold object of making the test still more reliable and securing uniformity of results.

In this report our work is not put forward with any claim to finality, but for the purpose of inviting criticism and discussion.

From our investigation of the behaviour under different experimental conditions of each reagent employed in the Wassermann reaction, we have been led to formulate a routine method which we think is worthy of trial. In order to focus attention at the outset upon the practical issues involved, we have outlined this method in the first section of our report; we have then proceeded to discuss in detail the scientific data which, in our opinion, provide a justification for the technique proposed.

Throughout our work we have been in close touch with Brevet-Colonel Harrison, D.S.O., K.H.P., whom we wish to thank for the constant assistance of his advice, encouragement, and criticism.

Outline of Proposed Method.

Principles.—The essential principles involved are (1) the use of highly diluted antigen and (2) prolonged contact (18 hours) of antigen, patient's serum and complement at the temperature of the ice-chest.

Measurements.—All ingredients in the test are employed in equal volumes; the volume chosen is 0·25 c.c., measured by means of mercury-calibrated teat pipettes standardised so as to deliver this quantity when the fluid is discharged at a constant rate. The total volume in the test and control tubes during the fixation stage is 0·75 c.c. and in the hæmolytic stage 1·0 c.c.

Apparatus.—Test-tubes, 3-inch × ½-inch, washed in acid followed by distilled water and sterilised, inverted in wire baskets, in the hot-air steriliser at 170° C. for one hour.

Copper racks to take 12 tubes, to fit the water-bath.
Ice-chest to maintain T° of fluid in test-tubes below 6° C.
Water-bath to maintain T° at 37°–40° C.
Water-bath to maintain T° at 56° C.
Teat pipettes delivering 0·25 c.c. and 0·1 c.c.
1 c.c. pipettes graduated in hundredths.
Graduated pipettes and cylinders.

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* *See* Reports of the Medical Research Committee, Nos. 14 and 21.

Ingredients.—Normal saline prepared by dissolving 0·85 per cent. chemically pure sodium chloride in fresh glass-distilled water followed by filtration and sterilisation at 120° C. in the autoclave.

Human serum diluted 1 in 5 with normal saline and then heated for half an hour at 56° C.

Complement obtained by bleeding male guinea-pigs and used the same day, diluted 1 in 25 with normal saline.

Antigen prepared by adding normal saline quickly to alcoholic solution of acetone-insoluble lipoids to which alcoholic solution of cholesterin has been added in the requisite amount; the resulting suspension of lipoids should be clear or faintly opalescent, and should contain 1 part of the mixture of alcoholic solutions to 640 parts of normal saline.

Blood cells obtained from sheep's blood, collected in citrate solution, washed free from serum, formolised and made up with normal saline so that 6 per cent. of the sediment is present in the suspension.

Hæmolytic immune serum obtained from horses injected with sheep's blood cells.

Sensitised blood cell suspension prepared by mixing equal quantities of the 6 per cent. blood cell suspension and normal saline containing sufficient of the hæmolytic immune serum to give maximum sensitisation.

Order of Work.—Preliminary Steps:—

(1) Human sera to be pipetted from clot, centrifuged if necessary, then 0·25 c.c. to be mixed with 1 c.c. of normal saline and heated for half an hour at 56° C. in the water-bath.

(2) Guinea-pig to be bled, serum pipetted off, centrifuged, and diluted 1 in 25 with normal saline.

(3) Sensitised blood cell suspension prepared and kept at room T° in sufficient amount for the preliminary titration, *e.g.*, 0·25 c.c. sediment + 3·9 c.c. normal saline + 4·1 c.c. of the dilution of hæmolytic immune serum.

(4) Preliminary titration of complement (to ensure that it is of normal lytic activity).

(5) Preparation of antigen suspension maintained at 0° C. in ice-bath.

Setting up of Test and Controls. Fixation stage:—

Arrangement of tubes.

(*a*) Three tubes for each serum to be set one behind the other, and two sets of four tubes for the complement controls.

Addition of diluted human serum.

(*b*) Add 0·25 c.c. normal saline to each tube of the third row, then 0·25 c.c. of the appropriate diluted human serum to each of the three tubes arranged for its test. Remove 0·25 c.c. of the mixture from

the tube in the third row; thus for each serum there are two tubes containing 1 in 5 serum and one, that in the third rack, containing 1 in 10.

(*c*) Add 0·25 c.c. of normal saline to all tubes in the first row, which represents the serum control.

(*d*) Add 0·25 c.c. of complement diluted 1 in 25 to all tubes except the two sets of complement controls (*see* (*a*)).

Complement control sets.

(*e*) Add to the four tubes of each complement control set 0·25 c.c. of complement diluted 1 in 25, 1 in 31, 1 in 42, and 1 in 62, corresponding to 0·01, 0·008, 0·006, 0·004 c.c. of neat guinea-pig serum. (The further dilutions of the 1 in 25 complement are conveniently made by taking three tubes and adding to the first 0·25 c.c. of normal saline, to the second 0·5 c.c., to the third 0·75 c.c. (*i.e.*, one, two and three volumes), and then mixing with four, three and two volumes of the 1 in 25 dilution of complement).

(*f*) Add to each of the first set of four tubes (complement control) two volumes of normal saline (0·5 c.c.); add to each of the second set (control of antigen on complement) one volume (0·25 c.c.) of normal saline.

Addition of antigen in the cold.

(*g*) Cool all tubes by keeping racks in ice-chest for one hour.

(*h*) Add cooled antigen suspension (one volume) to all tubes except the first set of four controls and the first (serum control) row ((*c*) above).

(*i*) Place in ice-chest for 16 to 24 hours (*i.e.*, overnight).

Hæmolytic Stage.—Next day :—

(1) Prepare sufficient 3 per cent. sensitised cell suspension.

(2) Remove racks from ice-chest and add 0·25 c.c. of this suspension to all tubes (the complement control sets last in order).

(3) Place in water-bath at 37° to 40° C.

(4) After 15′ take out each rack, shake and replace.

(5) After 30′ take out each rack, shake and replace.

(6) After 60′ take out each rack, shake and place at room T° for three hours.

(7) Read results, noting first the control tubes.

Reading of Results :—

(*a*) *Controls.*—Hæmolysis should be complete (1) in the second tube (*i.e.*, that containing 1 in 31 dilution of complement) in both sets of complement controls (*i.e.*, with and without antigen), and (2) in the first tube (*i.e.*, the serum control tube) for each serum.

(*b*) Reactions.—(i) A positive reaction in cases for diagnosis is then represented by complete absence of hæmolysis in the second tube, that containing the 1 in 5 dilution of patient's serum, and by slight hæmolysis at most in the third tube, viz., that containing the 1 in 10 dilution. A trace of hæmolysis in the second tube (1 in 5 dilution) may be neglected if, as often happens in such cases, the third tube (1 in 10 dilution) shows complete inhibition.

(ii) Complete lysis in both these tubes indicates a negative reaction.

(iii) Partial lysis in these two tubes should not be regarded as positive in a case for diagnosis, but should be taken into account in considering the need for continued treatment of a known case of syphilis.

In the sections which follow, further details are given regarding the method of procedure outlined above, together with the experimental evidence upon which its choice is based.

The Hæmolytic System.

(1) *Preparation of Constituents* :—

(*a*) Sheep's blood cells have been used throughout our experiments. The blood was obtained from the slaughter-house, and was caught as aseptically as possible in sterile bottles containing about one-fifth of their volume of sterile saline + 5 per cent. sodium citrate. It is preferable to place the bottle immediately on ice. As soon as possible after collection the blood cells were thoroughly washed by means of a large electric centrifuge. The final deposit was packed as firmly as possible by prolonged centrifuging; it was then measured and shaken up with formalin (0·1 c.c. of 1 in 10 dilution for every 5 c.c. of sediment) as recommended by Armand Delille.

These formolised cells keep perfectly for at least a week and at the end of that time show much less spontaneous hæmolysis than unformolised cells which have been kept in the ice-chest for 24 to 48 hours.

If care is taken to measure in the same way on each occasion, cell suspensions of remarkable uniformity can be obtained. Our practice has been to measure the required quantity of sediment with a 0·5 c.c. pipette previously wetted with saline; to discharge this quantity into the required amount of saline and then to wash out the pipette in the cell suspension thus prepared.

We have not met with abnormally resistent or fragile specimens of blood as measured by their behaviour in the hæmolytic reaction.

(*b*) The hæmolytic immune serum we used was horse *v.* sheep, and was obtained from Burroughs, Wellcome & Co. It has always been of high titre, and has never been used in greater concentration than 1 in 250 in the sensitised suspension.

(*c*) To prepare the sensitised cells a 6 per cent. suspension of the blood sediment was rapidly mixed with an equal quantity of the appropriate dilution of the hæmolytic immune serum, the latter being added to the former. The sensitised suspension was then allowed to stand for 15 minutes at room temperature before use.

(2) *Conditions of Lysis.*—For lysis of the cells, incubation was carried on in a water-bath at 37° to 40° C.; the racks containing the tubes were taken out and shaken after 10′, 30′ and finally at the end of an hour; thereupon the racks were removed from the bath and left at room temperature till the intact cells settled sufficiently to allow recognition of any tinting of the supernatant fluid.

(3) *Notation.*—In Tables I. and II., as in all other tables illustrating experiments in this report, the following signs are used in describing lysis.

0 = supernatant fluid quite colourless.
tr = slight but definite tinting with hæmoglobin.
? tr = less definite tinting.
\+ = approximately $\frac{1}{5}$th of the cells are lysed.
\++ = ,, $\frac{2}{5}$ths ,, ,,
\+++ = ,, $\frac{3}{5}$ths ,, ,,
\++++ = ,, $\frac{4}{5}$ths ,, ,,
c = complete lysis of the cells.
? c = almost complete lysis, but fluid not quite perfectly clear on shaking.

(4) *Titration of Hæmolytic Immune Serum.*—The method of titration of the hæmolytic immune serum is shown in the left half of Table I. (pp. 15–17). The object was to determine the dilution which gave maximum sensitisation of cells. Samples of fresh complement from different guinea-pigs were taken and distributed in several similar sets of graded amounts. Cells sensitised with graded dilutions of the immune serum were added to the different sets. It will be seen that maximum hæmolytic activity was reached for all the complements with cells sensitised with 1 in 250 dilution of the immune serum under test (*i.e.*, 6 per cent. cells and 1 in 125 H.I.S.).* This

* Tested in the ordinary way with excess of complement this sensitisation is equivalent to ten minimum hæmolytic doses of this particular serum.

represents, therefore, the point of "maximum sensitisation"; increase in the amount of hæmolytic immune serum beyond this produced no further diminution in the amount of complement necessary for complete lysis; it may, on the contrary, though this does not appear in the Table, actually increase the amount of complement necessary for complete lysis, possibly in virtue of the serum colloid reaching an amount sufficient to interfere with the lytic activity.

(5) *Reasons for choice of Maximum Sensitisation.* — In several methods, including the original technique of Wassermann, maximum delicacy is not aimed at in the reaction, and a surplus of hæmolytic activity over and above the minimum necessary to lyse the test corpuscles is obtained by using twice the minimum amount of hæmolytic serum while keeping to the standard amount of complement with which the hæmolytic unit was estimated. As this quantity of hæmolytic serum is usually insufficient to give maximum sensitisation, its employment may exaggerate the variations in hæmolytic activity of different complements, since some complements exert their maximum lytic activity with lower degrees of sensitisation of the cells than others. It is impossible to forecast for a given complement the effect on its lytic activity of sensitising with a given multiple of the unit. In consequence, complements which were partially destroyed to the same degree in a Wassermann reaction might give different results on application of the hæmolytic system, according as they were capable of acting in low concentration or not with cells sensitised to a lower degree than the maximum.

When one aims at making the test as delicate as possible, the degree of sensitisation assumes greater importance.

There are two methods to consider: (1) the cells may be highly sensitised, so that the complement will be able to exert its lytic action under the most favourable circumstances, or (2) a limited sensitisation may be employed, *e.g.*, by using not more than one or two of the units of hæmolytic serum which will give complete lysis of the test cells in the presence of excess of complement. In each case one might expect to obtain maximum delicacy in the Wassermann reaction by employing an amount of complement just sufficient to ensure complete lysis in the test under all circumstances, except those giving rise to the specific reaction. The matter is not so simple, however, owing to the fact that the using up of complement in the Wassermann reaction is not expressible in terms of hæmolytic activity only, but also in terms of the amount of guinea-pig serum present. In the first method the minimum lytic amount of complement is contained in a small quantity of guinea-pig serum, while in the second the quantity of guinea-pig serum must be larger; *e.g.*, with "minimal" sensitisation it may be five or more times as large as in the first method.

Hence the effect of participation in a Wassermann reaction with equal amounts of syphilitic serum and antigen is very different in the two cases. If, in the first case, the resulting diminution in the lytic activity of the complement is just equivalent to complete abolition, *i.e.*, all the cells remain unlysed, in the second case degrees of lysis from $\frac{1}{5}$th to $\frac{4}{5}$ths of the cells may remain. Though in the second case there is, as in the first, just one hæmolytic unit, yet this unit of activity is carried by a much larger quantity of guinea-pig serum and, as it is this guinea-pig serum as a colloid which is affected in the Wassermann reaction, a much more powerful reaction, *i.e.*, much greater quantities of syphilitic serum (or of specific Wassermann substance) and antigen would be required in order to abolish completely its hæmolytic activity. This is a strong reason for preferring (1), the method of maximum sensitisation.

Further observations on Tables I. and II.—To save repetition, Table I. is utilised also to compare complements as regards hæmolytic strength (left half) and fixability (right half). It shows that when the complements differed from each other in hæmolytic activity the reduction in lytic power which resulted from their participation in a Wassermann reaction bore no constant relation to their original hæmolytic strength. For example, complement No. 52, of which 0·15 c.c. of the 1 in 25 dilution gives complete lysis with the maximal sensitisation, produces in the W.R. with the 1 in 80 dilution of syphilitic serum a ? trace of lysis. The stronger complement No. 91, of which the lytic dose under the same conditions is 0·12 c.c., gives rather less lysis with this dilution, *i.e.*, a rather stronger W.R., while the weak complement, No. 93, of which more than 0·2 c.c. is required for complete lysis gives distinctly more lysis in the W.R., *i.e.*, gives a weaker positive result. Complement No. 95, which is approximately equal in lytic activity to No. 52, gives on the other hand a trifle more lysis in the W.R.

Table I. is followed by Table II. to demonstrate the effect of freezing complement. It is seen that freezing for three days had very slight effect on the hæmolytic activity except in the case of the weak complement, No. 93, which became still weaker; on the other hand, in all cases the frozen complements showed greater reduction in lytic strength as the result of the Wassermann reaction, *i.e.*, they became more easily fixed. Taking, for example, the W.R. given by the 1 in 80 dilution of syphilitic serum, it will be observed that all the frozen complements fail to give any trace of lysis, whereas with the same complements unfrozen distinct traces of lysis were recorded.

TABLE I.

A.—Estimation of Dilution of Hæmolytic Immune Serum giving Maximum Sensitisation of Red Cells.

B.—Estimation of Fixability of Complement.

For titration the complements were distributed in the graded quantities on the day of collection, and the dilutions were placed in the ice-chest for 18 hours before the addition of the sensitised red cells in 3 per cent. suspension.

Dilution of H. I. Serum mixed with 6 per cent. cells.	A.—Complement Titration. Quantities of 1 : 25 Complement in c.c.					B.—Complement Fixation Experiment.—Ice-chest (18 hours). 1 vol. of 1 : 25 Complement. 1 vol of 1 : 640 Antigen. 1 vol. of each Serum Dilution. Dilutions of a Positive Serum (No. 14) as under.				
	·3	·25	·2	·15	·1	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640
1 : 1000 - - -	+++	+++	++	++	+	0	0	tr	++	+++
	·2	·15	·12	·1	·08					
1 : 500 - - -	c	? c	++++	+++	++	0	0	+	+++	++++
1 : 250 - - -	c	c	++++	++++	++	0	? 0	+	++++	? c
1 : 125 - - -	c	c	? c	++++	+++	0	? tr	++	c	c
1 : 62·5 - - -	c	c	? c	++++	+++	0	? tr	+++	c	c

TABLE I.—*continued.*

A.—Estimation of Dilution of Hæmolytic Immune Serum giving Maximum Sensitisation of Red Cells—*continued.*

B.—Estimation of Fixability of Complement—*continued.*

Dilution of H. I. Serum mixed with 6 per cent. cells.	A.—Complement Titration. Quantities of 1 : 25 Complement in c.c.					B. - Complement Fixation Experiment.—Ice-chest (18 hours). Complement. G. Pig. 90. 1 vol. of 1 : 25 Complement. 1 vol. of 1 : 640 Antigen. 1 vol. of each Serum Dilution. Dilutions of a Positive Serum (No. 14) as under.				
	·3	·25	·2	·15	·1	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640
1 : 1000 - - -	++++	+++	++	+	tr	0	0	+	? c	? c
	·2	·15	·12	·1	·08					
1 : 500 - - -	c	c	? c	++++	+++	0	? 0	++++	? c	c
1 : 250 - - -	c	c	c	++++	+++	0	? tr	++++	c	c
1 : 125 - - -	c	c	c	? c	++++	0	tr	? c	c	c
1 : 62·5 - - -	c	c	c	? c	++++	0	tr	? c	c	c

Complement. G. Pig 91.

	·3	·25	·2	·15	·1					
1 : 1000 - - -	++++	++++	+++	++	+	0	0	...	++++	? c
	·2	·15	·12	·1	·08					
1 : 500 - - -	c	c	? c	++++	++	0	0	...	? c	c
1 : 250 - - -	c	c	? c	++++	+++	0	0	...	c	c
1 : 125 - - -	c	c	c	? c	++++	0	? 0	...	c	c
1 : 62·5 - - -	c	c	c	? c	++++	0	0	...	c	c

Complement.
G. Pig 93.

	·3	·25	·2	·15	·1	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640
1 : 1000 - - -	++	+	tr	? 0	0	0	0	0	tr	+
	·2	·15	·12	·1	·08					
1 : 500 - - -	++++	++	tr	? tr	0	0	0	tr	+	+++
1 : 250 - - -	++++	+++	++	tr	? 0	0	? 0	tr	+	++++
1 : 125 - - -	? c	+++	++	+	tr	? 0	tr	+	++	? c
1 : 62·5 - - -	? c	++++	+++	++	+	? tr	tr	+	++++	? c

Complement.
G. Pig 95.

	·3	·25	·2	·15	·1					
1 : 1000 - - -	++	+	+	tr	0	0	0	+	++	++++
	·2	·15	·12	·1	·08					
1 : 500 - - -	c	? c	+++	++	+	0	? 0	+	? c	c
1 : 250 - - -	c	? c	++++	+++	++	0	? tr	++	? c	c
1 : 125 - - -	c	c	++++	+++	++	0	tr	++++	c	c
1 : 62·5 - - -	c	c	++++	+++	++	0	tr	? c	c	c

TABLE II.

REPETITION of EXPERIMENT I. with the same COMPLEMENTS which had been kept FROZEN SOLID for THREE DAYS.

Dilution of H. I. Serum mixed with 6 per cent. Cells.	A. Complement Titration. Quantities of 1 : 25 Complement in c.c.					B. Complement Fixation Experiment.—Ice-chest (18 hours). Complement. G. Pig. 52. 1 vol. of 1 : 25 Complement. 1 vol. of 1 : 640 Antigen. 1 vol. of each Serum dilution. Dilutions of a Positive Serum (14) as under.				
	·5	·45	·35	·3	·25	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640
1 : 1000 - - -	? c	? c	? c	? c	? c	0	0	? tr	++	? c
	·25	·2	·15	·12	·1					
1 : 500 - - -	c	c	? c	++++	+++	0	0	tr	++++	c
1 : 250 - - -	c	c	c	? c	+++	0	0	+	++++	c
1 : 125 - - -	c	c	c	? c	++++	0	0	+	? c	c
1 · 62·5 - - -	c	c	c	? c	++++	0	? tr	+	c	c

Complement.
G. Pig 90.

	·5	·45	·35	·3	·25	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640
1 : 1000 - - -	c	c	? c	? c	? c	0	0	? tr	+++	? c
	·25	·2	·15	·12	·1					
1 : 500 - - -	c	c	? c	++++	+++	0	0	tr	++++	c
1 : 250 - - -	c	c	c	? c	+++	0	0	tr	++++	c
1 : 125 - - -	c	c	c	? c	++++	0	0	+	? c	c
1 : 62·5 - - -	c	c	c	? c	? c	0	0	+	c	c

Complement.
G. Pig 91.

	·5	·45	·35	·3	·25					
1 : 1000 - - -	c	c	c	c	c	0	0	0	++	c
	·25	·2	·15	·12	·1					
1 : 500 - - -	c	c	c	? c	++++	0	0	? tr	? c	c
1 : 250 - - -	c	c	c	? c	? c	0	0	tr	++	c
1 : 125 - - -	c	c	c	? c	? c	0	0	+	? c	c
1 : 62·5 - - -	c	c	c	? c	? c	0	0	+	c	c

TABLE II.—*continued.*

Repetition of Experiment I. with the same Complements which had been kept Frozen Solid for Three Days—*continued.*

Dilution of H. I. Serum mixed with 6 per cent. Cells.	A. Complement Titration. Quantities of 1 : 25 Complement in c.c.					B. Complement Fixation Experiment.—Ice-chest (18) hours. 1 vol. of 1 : 25 Complement. 1 vol. of 1 : 640 Antigen. 1 vol. of each Serum dilution. Dilutions of a Positive Serum (No. 14) as under.				
						Complement G. P. 93.				
	·5	·45	·35	·3	·25	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640
1 : 1000 - - -	? c	++++	+++	+	tr	0	0	0	? 0	tr
	·25	·2	·15	·12	·1					
1 : 500 - - -	+	+	tr	? tr	? 0	0	0	0	? tr	+
1 : 250 - - -	+++	++	+	...	? tr	0	0	0	tr	+
1 : 125 - - -	++++	++	+	+	tr	0	0	? tr	tr	+
1 : 62·5 - - -	++++	++	+	+	tr	? 0	? 0	tr	tr	+
						Complement. G. Pig 95.				
	·5	·45	·35	·3	·25					
1 : 1000 - - -	c	c	c	? c	++++	0	0	? tr	++	? c
	·25	·2	·15	·12	·1					
1 : 500 - - -	c	c	? c	+++	++	0	0	+	++++	c
1 : 250 - - -	...	c	c	? c	+++	0	0	+	? c	c
1 : 125 - - -	c	c	c	? c	++++	0	? 0	+	c	c
1 : 62·5 - - -	c	c	c	? c	++++	0	? 0	+	c	c

Complement.

(1) *Source.* — Guinea-pig complement has been used exclusively in our experiments. The blood was placed immediately in the incubator at 37° C. for half-an-hour, after which the clot was usually found retracted and the serum ready to be pipetted off.

(2) *Variability of Complement.*—Of all the reagents in the W.R., complement is the least capable of standardisation, and it is certainly the reagent which is most often responsible for discrepancies in the results on different occasions. It has been suggested that by pooling the sera from a number of guinea-pigs a complement of average character might be obtained. This is probably true so far as the hæmolytic activity is concerned, but it is not the case as regards the behaviour in the Wassermann reaction, *see* Table III. (p. 25). It will be observed, for example, that the readings for the W.R. with the 1 in 20 dilution of the syphilitic serum vary with the different mixtures of complement from ++ lysis to ? trace lysis : the range of variation is not much less than that shown by the individual complements.

Variations in hæmolytic activity in the sera of well-fed guinea-pigs are of less frequent occurrence, and are of less importance in the test, than variations in the degree to which the complement is inhibited in the presence of antigen alone or, still more important, in presence of the mixture of antigen and human serum.

It is necessary, therefore, to study the differences of this character between the complements of different guinea-pigs and to form an opinion as to their influence on the result of the W.R. For this purpose repeated observations on the same guinea-pig are more valuable than single examinations. The following data have been obtained by the study of 60 guinea-pigs from which blood was removed at successive intervals.

(a) *Variations in Hæmolytic Activity.*—Healthy male guinea-pigs, with few exceptions, furnish complement which varies in hæmolytic activity only within well-defined and narrow limits. The minimum concentration of complement necessary for complete lysis in our experiments has lain between 1 in 240 and 1 in 160, and has most commonly been 1 in 200. The latter figure is obtained thus : one volume (0·25 c.c.) of a 1 in 50 dilution of complement is added to two volumes (0·5 c.c.) of salt solution and one volume (0·25 c.c.) of a 3 per cent. suspension of fully sensitised sheep's cells (*i.e.*, 1 in 50 complement diluted 1 in 4 = 1 in 200). Any complement which requires a greater concentration than 1 in 125 to effect complete lysis under the above conditions should not be employed.

The conditions which we propose for the actual W.R. involve keeping the complement diluted in the ice-chest overnight before

the sensitised cells are added. After this period it was generally found that the hæmolytic activity, as compared with that on the day of withdrawal, had not depreciated; occasionally, however, an unusually active complement appeared to return more nearly to the average after being kept in the diluted state in the ice-chest. Incubation in the water-bath at 37° C., on the other hand, often exerts a definitely depreciatory influence on the hæmolytic activity of complement; when graded quantities are compared, although the actual minimum concentration required for complete lysis is not changed, the transition from the completely effective to the completely ineffective dose becomes more abrupt than is the case with fresh complement which has not been incubated at 37° C. before the addition of the sensitised cells, or with complement which has remained diluted in the ice-chest overnight. For example, two sets of complement titrations were compared, one of which was kept at room temperature while the other was incubated for an hour at 37° C. before the addition of a volume of sensitised cells; the results are given below:—

Complement Dilution.	Saline.	Lysis. After one hour at 37°.	
		Incubated at 37° C.	Room Temperature.
(1 volume).	(2 volumes).		
1 in 25 - - - - -	„	c	c
1 in 31 - - - - -	„	c	c
1 in 42 - - - - -	„	tr	? c
1 in 62 - - - - -	„	0	++++

The complement furnished by pregnant guinea-pigs or those which had recently had young was, as a rule, distinctly weaker than normal in hæmolytic activity, and in many cases the concentration required in the lytic experiments was five times as high as the average.

A single experiment was made upon the influence of diet upon the activity of complement. A set of six guinea-pigs was fed exclusively upon green food, which is inadequate to maintain them in good health. Although at the end of a month they had become very thin, their complements did not show any appreciable diminution in lytic power as contrasted with that at the beginning of the experiment.

(b) *Variations in Sensitiveness to the Inhibitory Action of Antigen alone.*—It is well known that guinea-pig complements differ in the degree to which they are weakened or destroyed by contact with antigen in salt solution. These differences, as will be shown later, are much more pronounced with certain kinds of antigen. Among the 60 guinea-pigs, the complements of which were tested on more than one occasion, four (males) were

found to yield complements which were hypersensitive to the action of a cholesterinised antigen and were therefore regarded as unsafe for use in a Wassermann test. In these four cases this characteristic was found to be persistent on repeated examinations during a full year. An autopsy made upon one failed to reveal any evident disease. Others showed a moderate degree of sensitiveness which varied on different occasions. The majority of complements showed only very slight depreciation in lytic power after incubation with cholesterinised antigens, and a few appeared to be quite unaffected.

As regards the influence of pregnancy, not only may the hæmolytic activity be lower than normal, as mentioned above, but this reduction is often greatly exaggerated by the presence of antigen. For this reason we have recommended that only complement from male guinea-pigs should be used in a Wassermann test; even very early pregnancy may be associated with increased sensitiveness of complement, and hence strict segregation of females would be necessary if they were to be used as a source of complement.

The cause of abnormal sensitiveness to the action of antigen is not fully understood, although it has been thought to be most probably related to the precipitability of the serum globulins. It has been suggested that the phenomenon of complement fixation in the presence of the specific antigen-serum complex is also the result of a precipitation of the complement-bearing globulins. Thus the principle of the two reactions, the inhibition of complement by antigen and by the specific complex, would be the same. In favour of this supposition is the fact that certain conditions affect both sensitiveness to antigen and specific fixability in the same way; thus both are exaggerated when the antigen contains considerable quantities of cholesterin, and in both cases the weakening or destructive effect on complement is diminished in the presence of a high concentration of serum proteins; moreover complements of unusual sensitiveness to antigen alone generally exhibit increased specific fixability.

On the other hand, we have made some observations which seem inconsistent with the supposition of identity in principle. In relatively rare instances a complement which is abnormally sensitive to antigen alone may actually be less fixable in the specific reaction, *i.e.*, give a weaker Wassermann reaction when compared with an insensitive complement used in the same amount. In such cases sensitiveness to antigen and specific fixability do not run parallel. A possible explanation may be found in the complicating factor introduced by the inactivated human serum acting as an inert colloid, which in greater or less degree protects complements against the action of antigen alone, but does not interfere to the same extent with their natural differences in fixability in the specific reaction.

(3) *Effect of variability of Complement on the Result of the W.R.*—When the sera of two guinea-pigs differ in hæmolytic activity, it is unsafe to assume that this difference can be compensated for by using less of the stronger or more of the weaker, because the readiness with which each is fixed has to be taken into account. In some instances, it is true, the more active complement may show more tendency to lysis than the less active when the two are used in equal quantities for a Wassermann reaction, *i.e.*, the use of the former may result in a weaker positive; in such instances the two complements, though unequal in hæmolytic value, have apparently an equal proportion of their hæmolytic activity abolished by the Wassermann reaction, *i.e.*, they are equally readily fixed. But, as shown in Tables IV. to VIII. (pp. 29–35, and *vide also* Tables I. and II.), this is not always the case. The more active complement may be more readily fixed and give a stronger positive than the less active when the two are used in equal amounts. In such instances the attempt to compensate by using less of the stronger complement would naturally increase the divergence of the results. A similar objection must be raised against attempts to compensate for differences in sensitiveness to antigen by quantitative adjustment. For not only (1) do human sera vary in their power to diminish this sensitiveness to antigen, but also, as was mentioned above, (2) a complement which is sensitive to antigen alone may not be fixed with corresponding ease by the specific antigen-serum mixture.

TABLE III.

Effect of mixing Unselected Complements.

No. of Complement.	Complement Titration.				Complement titrated +1 : 40 Antigen 3 : 2 Cholesterin.		Wassermann Reaction (ice-chest). 1 vol. of 1 : 25 Complement. 1 vol. of 1 : 40 Antigen. 1 vol. of Serum 10 diluted.		
	1 : 30	1 : 40	1 : 50	1 : 60	1 : 25	1 : 50	1 : 5	1 : 10	1 : 20
7	c	c	c	? c	c	c	0	0	tr
*8	? c	++	+	+	? 0	0	0	0	0
77	c	c	c	? c	tr	tr	0	0	? 0
302	c	c	c	c	c	c	? 0	tr	+++
303	c	c	c	c	c	c	? 0	+	? c
305	c	c	c	? c	++	+	0	0	? tr
1	c	c	c	c	c	c	? 0	tr	++
2	c	c	c	++++	? c	+++	0	0	? tr
3	c	c	c	? c	c	++	0	? tr	+

* This G.-P. was afterwards found to be a female which had recently had young (symphysis pubis relaxed).

Nos. 7 to 305 = single fresh complements.
„ 1 = mixture (equal parts) of 302, 303 and 305.
„ 2 = mixture (equal parts) of 7, 8, 77, 302, 303 and 305.
„ 3 = mixture (equal parts) of 77, 302, 303 and 305.
It is evident that mixtures are not necessarily more likely to give concordant results than single complements.

In the following Tables IV. to VIII. different complements are compared in respect of (1) hæmolytic activity, (2) sensitiveness to antigen alone, and (3) capacity for being fixed by antigen in the presence of a positive serum.

In Table IV. five fresh complements and a pooled mixture of equal parts of the five are compared. In hæmolytic activity the differences are slight, No. 76 being the weakest. The plain extract without added cholesterin has very little effect on any. No. 76, which has the lowest hæmolytic value, is the most sensitive to cholesterinised extract; the rest are only slightly sensitive. Comparing the specific fixation experiments with human sera Nos. 12, 8, 10, 4, 1, it will be seen that all complements give the same result in the case of the extract without added cholesterin, but that the cholesterinised extract produces divergent results with the different complements. The divergency is more marked with the strongly positive serum No. 12 than with the weaker No. 4; with the latter, indeed, lysis begins with the 1 in 40 dilution in the case of each complement. The explanation may partly be that in the case of the highly diluted No. 12 serum there is an insufficient amount of serum protein present to neutralise the inhibitory action of the cholesterin extract upon the complement; hence the apparent differences in the W.R. with different complements and No. 12 serum are partly due to the different sensitiveness of the complements to antigen alone.

Table V.—(*a*) The first experiment shows that, when using six complements differing in sensitiveness to antigen, and, to a slight extent, in hæmolytic activity, lysis begins in each case at the same dilution of a positive serum. The antigen consists of alcoholic heart extract saturated with cholesterin at ice-chest T°, *i.e.*, there is not an excessive amount of cholesterin.

(*b*) In the second experiment with six different complements the antigen is more concentrated (1 in 40 instead of 1 in 160) and contains 1 per cent. alcoholic solution of cholesterin in the proportion of 2 to 3 of extract. There is greater divergency in the results, and it is particularly to be noted that capacity to be inhibited by the specific combination does not correspond with the sensitiveness of the complement to the action of antigen alone; *cf.* No. 36 with 62 and 80; No. 36, a sensitive complement, gives less fixation with the W.R. than 62 and 80, the insensitive complements. This divergency persists whether a fixed dose of complement and varying quantities of patient's serum are used or whether, as in (*c*), the amount of serum is fixed and the quantities of complement varied.

In Table VI., Experiment (*a*), the two methods of estimating the strength of a positive serum are contrasted (1) by diluting the patient's serum, (2) by using increasing quantities of complement. It will be seen that the variability of complements is exaggerated by the latter method. Complement 57 furnishes

a useful example of the lack of concordance in the three properties which we have been discussing; it is of normal activity as regards its lytic titre; it is very insensitive to antigen; it is highly sensitive to the antigen-serum complex.

In Experiment (*b*) two complements were selected, equal in hæmolytic activity but differing in sensitiveness to antigen. A number of sera were tested, both strong and weak. It will be seen that, if one takes the first dilution of serum showing lysis, the results with the two complements are practically identical. It is evident from the uniform results obtained in the W.R. with the two complements that it is unnecessary to compensate for the greater anticomplementary effect of antigen on No. 79. A further point worth noting is that the protective action of the serum constituents against the inhibitory effect of the cholesterinised antigen tends to disappear at about 1 : 80 dilution. It was suggested above that this might partly explain why the variability of different complements is most marked when they are used to test a highly diluted strongly positive serum with a highly cholesterinised antigen.

A further experiment, Table VII., shows that when the strength of a W.R. is estimated by graded doses of complement, the idiosyncrasies of the different complements produce greater variations in the apparent strength of the result than when the estimation is made by diluting out the serum and using a single quantity of complement. For example, the W.R. with complements 52 and 57, which are equal in hæmolytic strength and only slightly different in sensitiveness to antigen alone, produces complete inactivation of a 1 in 6 dilution of the latter complement, but only of a 1 in 12 dilution of the former, *i.e.*, it fixes 0·04 c.c. of complement 57 and 0·02 c.c. of 52, whereas by the method of dilution of the syphilitic serum each produces a trace of lysis at the same (1 in 40) dilution.

Table VIII. shows that even with the latter method some irregularity persists. For example, the W.R. with the 1 in 40 dilution of the syphilitic serum yields readings varying from complete lysis to a ? trace of lysis with the different complements, although in these no marked difference in lytic strength or sensitiveness to antigen alone could be detected.

On the other hand, the regularity of the results obtained with an antigen which is not anticomplementary may be instanced by the experiment in Table IX. (p. 36) where a 1 : 20 dilution of a positive serum gave, with 10 different male guinea-pig complements and a "Noguchi" antigen cholesterinised to standard, practically identical results, *i.e.*, a tr or a ? tr of lysis.

(4) *Choice of quantity of Complement to be used in the W.R.* —The data which we have recorded show clearly that the method of adjusting the amount of complement so that it may represent an arbitrary standard of hæmolytic activity, both

alone and in the presence of antigen, cannot be relied upon to produce uniformity of results in the Wassermann reaction. Therefore we have proposed that, as a reagent in the test, the complement should be represented by a standard amount of fresh guinea-pig serum, no attention being paid, within certain limits, to its hæmolytic activity or to its sensitiveness to antigen alone; when these limits are exceeded in either case the complement is to be discarded.

In choosing the standard quantity of guinea-pig serum, the first and essential consideration must be that it should be capable in all circumstances of giving complete lysis in a test in which the human serum is derived from a non-syphilitic person. At the same time, the use of a quantity considerably in excess of the minimum complying with the above requirement involves a diminution in the delicacy of the reaction with consequent failure to detect certain cases of syphilitic infection; it also tends to exaggerate the inevitable variations in the result which depend upon inherent differences in the fixability of the complements. Such excess can only be justified on two grounds; (i) some non-syphilitic sera, under the influence of the antigen used, may possibly produce sufficient complement fixation to cause confusion, when a minimal lytic dose of complement is employed; or (ii) accidental errors, such as impurities in the glassware, want of accuracy in measuring and other imperfections in technique might lead to the appearance of false positives unless a margin of safety was provided.

The irregular sensitiveness towards antigen alone, though it is probably only exceptionally a special disturbing factor, we propose to obviate to a considerable extent by the use of an antigen which rarely shows anticomplementary properties.

The main safeguard against accidental errors should be the use of adequate controls; we do not consider it desirable to rely on excess of complement as a margin of safety for this purpose.

The conclusion we have reached is that, for the W.R. conducted as described, the standard quantity of complement, *i.e.*, of fresh serum from healthy male guinea-pigs, should be 0·01 c.c. added in the form of one volume (0·25 c.c.) of a 1 : 25 dilution. Our experience has been that, after excluding the rare specimens of complement which fail to pass the preliminary test (complete lysis on the addition of a volume of the 1 in 31 dilution), the above standard quantity has given complete lysis, *i.e.*, a frank negative, with all non-syphilitic sera. The standard exceeds the absolute minimum necessary by a slight and variable amount, but we do not think this margin is large enough to impair the delicacy of the test to any important extent. It has the advantage of enabling one to use the great majority of male guinea-pig complements irrespective of minor variations in hæmolytic activity; the latter have very little influence on the result of the Wassermann reaction, and are to be disregarded.

TABLE IV.

Comparison of Complements in respect of Hæmolytic Activity and Capacity to be inhibited by Antigen alone and by Antigen + a Positive Serum (Ice-chest Fixation).

No. of Complement.	Complement Titration.					Complement titrated + 1 vol. of 1 : 10 Plain Extract.					Complement titrated + 1 : 40 Cholesterinised Extract.			
	1 : 30	1 : 40	1 : 50	1 : 60	1 : 80	1 : 30	1 : 40	1 : 50	1 : 60	1 : 80	1 : 25	1 : 30	1 : 40	1 : 60
62	c	c	? c	++++	+++	c	c	++++	+++	++	c	c	++++	++
65	c	c	c	? c	++++	c	c	c	++++	++	c	c	c	++++
76	c	c	++++	++	+	c	? c	+++	++	tr	++	+	+	+
78	c	c	c	? c	+++	c	c	c	++++	++	c	c	c	++++
79	c	c	c	? c	++++	c	c	? c	++++	+++	c	c	? c	++++
Mixed	c	c	c	? c	+++	c	c	? c	++++	+++	c	c	++++	++++

No. of Complement.	1 vol. of 1 : 10 Plain Extract. 1 vol. of 1 : 25 Complement. 1 vol. of Serum 12 diluted as under.				1 vol. of 1 : 40 Cholesterinised Extract. 1 vol. of 1 : 25 Complement. 1 vol. of Serum 12 diluted as under.					1 vol. of 1 : 10 Plain Extract. 1 vol. of 1 : 25 Complement. 1 vol. of Serum diluted as under.			
										Serum 8.	Serum 10.		
	1 : 20	1 : 40	1 : 80	1 : 160	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 5	1 : 5	1 : 10	1 : 20
62	0	0	? tr	++++	0	0	0	0	+	+++	0	0	? tr
65	0	0	? tr	++++	0	0	0	tr	++	++	0	0	tr
76	0	0	? tr	++++	0	0	0	0	0	++	0	0	? tr
78	0	0	? tr	++++	0	0	0	tr	+++	+++	0	0	tr
79	0	0	? tr	++++	0	0	0	0	+	++	0	0	tr
Mixed	0	0	? tr	++++	0	0	0	0	tr	++	0	0	? tr

TABLE IV.—*continued.*

Comparison of Complements in respect of Hæmolytic Activity and Capacity to be inhibited by Antigen alone and by Antigen + a Positive Serum—*continued.*

No. of Complement.	1 vol. of 1 : 10 Plain Extract. 1 vol. of 1 : 25 Complement. 1 vol. of Serum diluted as under.							1 vol. of 1 : 40 Cholesterinised Extract. 1 vol. of 1 : 25 Complement. 1 vol. of Serum diluted as under.				
	Serum 4.				Serum 1.			Serum 4.				
	1 : 20	1 : 40	1 : 80	1 : 160	1 : 5	1 : 10	1 : 20	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320
62	0	tr	c	c	? 0	+	? c	0	tr	++	++++	? c
65	0	+	c	c	? 0	+	c	0	+	c	c	c
76	0	tr	? c	c	? 0	tr	? c	0	? tr	+	++	++++
78	0	tr	c	c	? 0	+	c	0	+	c	c	c
79	0	+	c	c	? 0	+	c	0	tr	+++	++++	? c
Mixed	0	tr	c	c	? 0	+	c	0	+	++++	c	c

TABLE V.

Comparison of Complements in respect of Hæmolytic Activity and Capacity to be inhibited by Antigen alone and by Antigen + a Positive Serum (Ice-chest Fixation).

Exp. (*a.*)

No. of Complement.	Complement Titration.				Complement titrated + 1 vol. of 1 : 160 Extract saturated with Cholesterin.			1 vol. of 1 : 160 Extract saturated with Cholesterin. 1 vol. of 1 : 25 Complement. 1 vol. of Serum diluted as under.				
	1 : 25	1 : 30	1 : 40	1 : 60	1 : 25	1 : 30	1 : 40	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320
55	c	c	c	+++	c	? c	++++	0	0	tr	...	...
56	c	c	c	++++	c	+++	++	0	0	...	++	++++
57	c	c	c	++	c	c	? c	0	0	tr	? c	c
59	c	c	c	? c	c	c	c	0	0	+	c	c
62	c	c	c	+	+++	++	tr	0	0	tr	++	++
63	c	c	c	++++	c	c	? c	0	0	tr	c	c

TABLE V.—*continued.*

Comparison of Complements in respect of Hæmolytic Activity and Capacity to be inhibited by Antigen alone and by Antigen + a Positive Serum—*continued.*

(*b.*)

No. of Complement.	Complement Titration.				Complement titrated + 1 vol. of 1 : 40 cholesterinised Extract.					1 vol. of 1 : 40 cholesterinised Extract. 1 vol. of 1 : 25 Complement. 1 vol. of Serum diluted as under.				
	1 : 40	1 : 50	1 : 60	1 : 80	1 : 15	1 : 25	1 : 30	1 : 40	1 : 60	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320
36 - - -	c	c	? c	? c	++	++	++	+	+	0	tr	+	++	++
62 - - -	c	c	? c	++++	c	c	c	? c	++++	0	0	tr	++	++++
77 - - -	c	? c	? c	++++	+	+	+	tr	tr	0	0	? 0	tr	+
78 - - -	c	c	c	? c	c	c	? c	? c	+++	0	? tr	+	++++	c
80 - - -	c	c	c	? c	...	c	c	c	c	0	0	tr	++++	c
83 - - -	c	c	c	? c	...	c	c	c	? c	0	0	+	? c	c

(*c.*)

No. of Complement.	1 vol of 1 : 40 cholesterinised Extract. 1 vol. of 1 : 5 Positive Serum. 1 vol. of each Complement diluted as under.				
	1 : 8	1 : 6	1 : 4	1 : 3	1 : 2 ·5
36 - - -	+	++++	c	c	c
62 - - -	tr	+	c	c	c
77 - - -	? 0	tr	++	c	c
78 - - -	+	++++	c	c	c
80 - - -	? tr	tr	++	c	c
83 - - -	tr	+	c	c	c

TABLE VI.

Prolonged contact in the Ice-chest.

Exp. *a*. Estimation of strength of reaction of a positive serum, (1) by dilution of the serum, (2) by increasing the amount of complement.

No. of Complement.	Complement titration.				Complement titrated + 1 vol. of 1 : 160 cholesterinised Extract.				1 vol. of 1 : 160 cholesterinised Extract. 1 vol. of 1 : 25 Complement. 1 vol. of Serum 5 diluted as under.					1 vol. 1 : 40 cholesterinised Extract. 1 vol. of Serum 5 diluted 1 : 5. Varying quantities of Complement as under.				
	1 : 30	1 : 40	1 : 50	1 : 60	1 : 25	1 : 30	1 : 40	1 : 50	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	·02 c.c	·03 c.c.	·04c.c.	·05c.c.	·06 c.c.
52	c	c	++++	++++	c	++++	+++	++	0	tr	+++	? c	? c	0	? 0	? tr	tr	++
55	c	c	? c	++++	c	? c	++++	+++	0	+	? c	c	c	0	tr	tr	++	++++
57	c	c	++++	+++	c	c	? c	++++	0	tr	? c	c	c	0	0	0	? tr	tr
76	c	c	? c	++++	c	c	++++	+++	0	+	? c	c	c	0	0	? tr	tr	++++
Mixed	c	c	? c	++++	c	? c	++++	+++	0	+	++++	? c	c	0	? 0	tr	+	++

Exp. *b*. Comparison of two complements.

No. of Complement.	Complement Titration.					1 vol. of 1 : 80 cholesterinised Extract.											
						1 vol. of 1 : 25 Complement 64. 1 vol. of Serum diluted as under.						1 vol. of 1 : 25 Complement 79. 1 vol. of Serum diluted as under.					
	1 : 30	1 : 40	1 : 50	1 : 60	1 : 80	Serum.	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320
64	c	? c	+++	++	tr											
79	c	? c	+++	++	? tr											
						906	+++	? c	c	c	c	+++	? c	? c	++++	++++
						908	tr	? c	c	c	c	tr	++++	? c	++++	++++
						909	0	0	0	? 0	++++	0	0	0	? 0	+
						910	tr	? c	c	c	c	tr	++++	? c	? c	++++
						914	0	0	tr	++++	c	0	0	tr	+	+
						915	0	0	0	tr	? c	0	0	0	? tr	+
						918	c	c	c	c	c	c	c	c	? c	++++
						919	? 0	++++	c	c	c	? 0	+++	? c	++++	++++
						927	++	c	c	c	c	+	++++	? c	++++	++++
	Complement titrated +1 : 80 cholesterinised Extract.															
	1 : 25	1 : 30	1 : 40	1 : 50	1 : 60											
64	c	c	++++	++	+											
79	++	+	tr	tr	tr											

TABLE VII.

Comparison of Complements.

Complement Titration with and without Antigen.

Number of Complement.	Complement Titration.					Complement titrated + 1 vol. of Extract 3 (0·4 per cent. Cholesterin) 1 : 160 dilution.				
	·25	·2	·15	·12	·1	·25	·2	·15	·12	·1
52 - - - -	c	c	c	++++	++++	c	++++	+++	++	+
55 - - - -	c	c	c	? c	++++	c	? c	++++	+++	+++
57 - - - -	c	c	c	++++	+++	c	c	? c	++++	+++
76 - - - -	c	c	c	? c	++++	c	c	++++	+++	++
Pooled - - -	c	c	c	? c	++++	c	? c	++++	+++	+++

Specific Fixation Test.

Fixation in Ice-chest overnight.

Number of Complement.	1 vol. of Serum 5, 1 : 5. 1 vol. of Extract 3, 3/2 with 1 °/₀ Cholest., 1 : 40. 1 vol. of each Complement diluted as under.					1 vol. of each Complement, 1 : 25. 1 vol. of Extract 3, 3/2 with 1 °/₀ Cholest., 1 : 160. 1 vol. of Serum 5 diluted as under.				
	1 : 12	1 : 8	1 : 6	1 : 5	1 : 4	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320
52 - - - -	0	? 0	? tr	tr	++	0	tr	+++	? c	? c
55 - - - -	0	tr	tr	++	++++	0	+	? c	c	c
57 - - - -	0	0	0	? tr	tr	0	tr	? c	c	c
76 - - - -	0	0	? tr	tr	+++	0	+	? c	c	c
Pooled - - -	0	? 0	tr	+	++	0	+	++++	? c	c

TABLE VIII.

Comparison of Complements.

Tested after standing overnight in the Ice-chest.

Designation of Complement.	Complement Titration.				Complement titrated + 1 vol. of Noguchi 1/1, with 1 % Cholesterin ; diluted 1 : 640.				Complement titrated + 1 vol. of R.R. 3/2, 1 : 160.			
	Amount of 1 : 25 Complement in Fractions of c.c.											
	·2	·15	·12	·1	·25	·2	·15	·1	·25	·2	·15	·1
A - - - -	c	c	c	? c	c	c	c	? c	c	c	c	? c
B - - - -	c	c	c	? c	c	c	c	++++	c	c	? c	++
C - - - -	c	c	c	? c	c	c	c	? c	c	c	c	? c
D - - - -	c	c	c	++++	c	c	c	+++	c	c	c	+++
E - - - -	c	c	c	? c	c	c	c	++++	c	? c	+++	tr
F - - - -	c	c	c	? c	c	c	c	? c	c	c	c	? c

Specific Fixation Test.

Designation of Complement.	Fixation at 37°, 1 hour. 1 vol. of Serum 15, 1 : 5. 1 vol. of Cholesterinised Noguchi Extract, 1 : 20. 1 vol. of each Complement diluted as under.					Fixation overnight in Ice-chest. 1 vol. of each Complement, 1 : 25. 1 vol. of Cholesterinised Noguchi Extract, 1 : 640. 1 vol. of Serum 15 titrated as under.				
	1 : 12	1 : 8	1 : 6	1 : 4	1 : 3	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160
A - - - -	? 0	++	c	c	c	0	tr	c	c	c
B - - - -	0	? 0	+	c	c	0	? tr	+++	c	c
C - - - -	0	+	? c	c	c	0	? tr	++++	c	c
D - - - -	0	0	? 0	tr	c	0	0	? tr	+++	c
E - - - -	0	? tr	tr	c	c	0	0	+	? c	c
F - - - -	? tr	+++	c	c	c	0	tr	c	c	c

TABLE IX.

Comparison of Ten Fresh Complements.

(All Male Guinea-pigs, otherwise unselected.)

No. of Complement.	Complement Titration.				Titration of Complement + 1 vol. 1 : 320 Noguchi Extract (2·5 Extract to 1 of 1 per cent. Cholesterin).				1 vol. 1 : 320 Noguchi Extract (2·5 Extract to 1 of 1 per cent. Cholesterin). 1 vol. Complement, 1 : 25. 1 vol. of Serum 9 diluted as under.				
	1 : 30	1 : 40	1 : 50	1 : 60	1 : 30	1 : 40	1 : 50	1 : 60	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80
1	c	c	c	? c	c	c	c	? c	? 0	tr	+	? c	c
2	c	c	c	? c	c	c	c	? c	0	? tr	+	? c	c
3	c	c	c	c	c	c	c	c	? tr	tr	+	c	c
4	c	c	c	c	c	c	c	c	0	? tr	tr	++++	c
5	c	c	c	c	c	c	c	c	0	? tr	+	c	c
6	c	c	c	? c	c	c	c	? c	0	? tr	tr	++++	c
7	c	c	c	? c	c	c	c	? c	? 0	? tr	+	c	c
8	c	c	c	? c	c	c	c	? c	0	? tr	tr	? c	c
9	c	c	? c	++++	c	c	c	++++	0	? tr	tr	+++	c
10	c	c	c	? c	c	c	c	? c	? 0	? tr	+	? c	c

Human Serum.

(1) *Preparation of Serum.*—The minimum amount necessary for test by the method proposed is 0·15 c.c., but for convenience in making the dilutions and as a reserve in case of accidents, about 0·5 c.c. should be available; this can usually be obtained from 1·5 c.c. to 2 c.c. of blood. The serum, which should not be deeply hæmoglobin stained, must be freed from blood cells either by centrifuging or by being allowed to stand in the ice-chest.

The serum should be so far as possible aseptic. Bacterial contamination often renders serum anticomplementary and therefore useless for the test by the ice-chest method of fixation. This method reveals anticomplementary properties which might escape notice, though probably equally undesirable, in the ordinary method of fixation.

(2) *Inactivation.*—There is no doubt that fresh serum, with certain antigens at least, may give a positive Wassermann reaction in the absence of syphilitic infection. For this reason it is generally agreed that the serum must be inactivated at 56° C. before use. As regards the length of time necessary for inactivation, we have not found that stronger reactions are given by our method after a short period of heating (10 minutes), than after the usual half-hour. Occasionally, indeed, the reaction has appeared to be weaker with the short period of inactivation, probably on account of the disturbing effect of the incompletely destroyed human complement. Our experiments show that a serum may be heated to a temperature of 56° C. for a much longer period than 30 minutes before its capacity to react is materially reduced. Exposure of serum to a temperature of 56° C. for four hours reduced its capacity to give a positive reaction only very slightly more than exposure for half an hour (Table X.). A serum which had been heated for half an hour at 56° C., and gave a positive reaction when diluted 1 in 160, was heated to 60° C. for a further period of 12 minutes. The reaction was reduced in strength, being obtained only in a dilution of 1 in 80, but no further reduction in strength occurred on continuing the heating at 60° C. for a full hour (Table XII.).

The slightly greater effect of heating to 66° C. upon two different sera, both strongly positive, is shown in Table X. At 70° C. (again Table X.) the positive reaction was rapidly weakened and disappeared completely after exposure for half-an-hour.

In the case of syphilitic cerebro-spinal fluid, while there was a slight diminution in reacting capacity after heating to 56° C. for 15 minutes, no further reduction occurred after an hour's exposure, and the positive reaction began to fail only at the end of two hours. Cerebro-spinal fluid gradually lost its reacting capacity as the result of exposure to a temperature of 62° C.; after 35 minutes at this temperature the fluid still

gave a weak positive reaction which disappeared altogether at the end of 45 minutes. (*Vide* Tables X. and XI.)

It is evident that the common procedure (that which we have adopted in this method), namely, inactivation at 56° C. for half an hour, is well within the limits of safety as regards any possible destruction of Wassermann reacting bodies; it might possibly with advantage be extended to one hour, as recently advised by Wassermann.

The usual practice in inactivation is to heat the serum in the undiluted condition. As regards the effect on the Wassermann reaction, we have found (Table XII.) that the sera inactivated in the diluted condition give equally strong or slightly stronger positive results when compared with the same sera inactivated before dilution.

The following is an experiment with an exceptional serum which became more anticomplementary as the temperature of inactivation was raised.

The serum dilutions and a volume of 1 : 25 complement were kept in contact overnight in the ice-chest before the addition of the sensitised cells.

Temperature and Duration of Inactivation.	Serum Dilutions.				
	1 : 2·5	1 : 5	1 : 10	1 : 20	1 : 40
Not heated - - -	c	c	c	c	c
56° ½ hour diluted -	c	c	c	c	c
„ undiluted -	++++	c	c	c	c
60° ¼ hour diluted -	+++	c	c	c	c
„ undiluted -	? tr	? tr	tr	++	+++
60° ½ hour diluted -	tr	c	c	c	c
„ undiluted -	? tr	? tr	tr	+	++

(3) *Preservation of Sterile Positive Serum.*—The capacity of syphilitic serum to react to the Wassermann test persists for several months provided the serum is kept sterile in sealed ampoules. There is, however, a gradual slow depreciation in the strength of the reaction; this is most marked during the first few weeks after collection.

In Tables XIII., XIV., XV. (pp. 42–46) are given examples of repeated tests upon sera which have been kept in sealed ampoules at room temperature. In the case of No. 6, excepting slight fluctuations due to variations in antigen and complement, there was practically no alteration in the capacity to react during the period from November 25th to March 3rd. The sera did not become anticomplementary.

It would appear from these results that strongly positive sera might be preserved as standards, as recommended by D'Este Emery, in order to control variations in the other constituents of the test.

TABLE X.

Effect of heating Syphilitic Serum on the Wassermann Reaction.

Ice-chest Fixation.

Exposure to 56° C.	1 vol. of 1 : 40 Noguchi ext. (no Cholesterin). 1 vol. of Serum 1 : 5 ; quantities of Complement as under.					1 vol. of 1 : 160 Noguchi ext. with 0·5 per cent. Cholesterin. 1 vol. of 1 : 25 Complement. 1 vol. of Serum 14 diluted as under.				
	0·02	0·04	0·06	0·08	0·1	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160
½ hour -	0	? tr	tr	+	c	0	0	++	c	c
1 „ -	...	...	...	...	...	0	? 0	++	c	c
2 hours -	0	? tr	tr	+	...	0	0	++	c	c
3 „ -	0	? tr	tr	+++	...	0	0	+++	c	c
4 „ -	0	? tr	tr	+++	...	0	0	+++	c	c

Exposure to 66° C. (after the usual preliminary inactivation).	1 vol. of 1 : 500 Noguchi ext. (0·5 per cent. Cholesterin). 1 vol. of Complement 1 : 25. 1 vol. of No. 12 Serum diluted as under (0·01 of inactivated G.-P. Serum added to each Serum Dilution).				
	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160
Not further heated.	0	0	0	0	+
10′ - - -	0	0	0	0	...
20′ - - -	0	0	0	0	...
30′ - - -	0	0	0	? tr	...
40′ - - -	0	0	0	tr	...
60′ - - -	0	0	0	+	...

Exposure to 66° C. after Preliminary Inactivation.	1 vol. of 1 : 640 Noguchi ext. (0·5 per cent. Cholesterin). 1 vol. of 1 : 25 Complement. 1 vol. of 14 Serum diluted as under.				
	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160
Not further heated.	0	0	0	? 0	tr
10′ - - -	0	0	? 0	++	c
30′ - - -	0	0	+	c	c
60′ - - -	0	? tr	c	c	c

TABLE X.—*continued.*

Exposure to 70° C. after Preliminary Inactivation.	1 vol. of 1 : 640 Noguchi ext. (0·5 per cent. Cholesterin). 1 vol. of 1 : 25 Complement. 1 vol. of 14 Serum diluted as under.				
	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160
Not further heated.	0	0	0	? 0	tr
10′ - - -	0	tr	? c	c	c
20′ - - -	++	c	c	c	c
30′ - - -	c	c	c	c	c

Exposure to 56° C.	1 vol. of 1 : 640 Noguchi ext. (0·5 per cent. Cholesterin). 1 vol. of 1 : 25 Complement. 1 vol. of Cerebro-spinal fluid diluted as under.									
	Heated before Dilution.					Heated after Dilution.				
	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32
15′ - -	0	0	0	? 0	tr	0	0	0	? 0	tr
40′ - -	0	0	0	? 0	tr	0	0	0	? 0	tr
60′ - -	0	0	0	? 0	tr	0	0	0	? 0	tr
120′ - -	0	0	? 0	? tr	tr	0	0	0	? tr	tr
Not heated -	0	0	0	0	? tr					

TABLE XI.

Effect of Heating Cerebro-spinal Fluid.

Exposure to 62° C.	1 vol. of Noguchi Extract, 0·25 per cent. Cholesterin. 1 vol. of 1 : 25 Complement. 1 vol. of Cerebro-spinal Fluid.					
	Undiluted.	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32
5′ - - -	...	0	0	0	+++	...
10′ - - -	...	0	0	tr	? c	c
15′ - - -	0	0	0	+	? c	c
20′ - - -	0	0	? tr	+++	c	c
25′ - - -	0	? tr	+	? c	c	c
35′ - - -	0	tr	+	? c	c	c
45′ - - -	c	c	c	c	c	c
Not heated -	...	0	0	0	tr	? c

The complement gave complete lysis with 0·15 of 1 : 25 dilution (a little less active after incubation at 37°). Antigen had no effect on the titre at 37° or at ice-chest temperature of fixation.

After 45′ heating to 62° C. in the presence of fresh normal human serum, 1 in 2 cerebro-spinal fluid (containing $\frac{1}{4}$ part of normal serum) still gave some inhibition when tested as above, *i.e.*, the serum protected it slightly against the effects of heating.

TABLE XII.

Effect of Heating Syphilitic Serum on the Wassermann Reaction continued.

Comparison of effect of Heating Diluted and Undiluted.

1 vol. of 1 : 80 Noguchi Extract (Cholesterin saturated).
1 vol. of 1 : 25 Complement.
1 vol. of Serum diluted as under.

Exposure to 60° C.	Heated before Dilution.					Heated after Dilution.					
	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	
12′ - - -	0	0	+++	c	c	...	0	++	c	c	Fixation at 37° for 45′.
20′ - - -	0	? 0	++++	c	c	...	? tr	++++	c	c	
30′ - - -	0	tr	? c	c	c	...	? tr	++++	c	c	
60′ - - -	0	++++	c	c	c	...	+	? c	c	c	
Inactivated at 56° for ½ hour only.	0	0	? 0	++	c						

	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	
12′ - - -	? 0	0	0	0	tr	...	...	0	0	0	tr	Ice-chest fixation over-night.
20′ - - -	? 0	0	0	0	tr	...	...	0	0	0	tr	
30′ - - -	? 0	0	0	0	tr	...	...	0	0	0	tr	
60′ - - -	? 0	0	0	0	++	...	...	0	0	0	tr	
Inactivated at 56° for ½ hour only.	0	0	0	0	0	tr	...					

Note.—The complement gave complete lysis with 0·15 c.c. of 1 : 25 dilution (a little less active after incubation at 37° C.). Antigen had no effect on the titre at 37° C. or at ice-chest temperature of fixation.

TABLE XIII.

REPEATED WASSERMANN TESTS upon a POSITIVE SERUM which, after INACTIVATION at 56° C. for half an hour, had been kept STERILE at ROOM TEMPERATURE in sealed ampoules. (Ice-chest Method.)

Titration of Positive Serum No. 1, bled 21.x.18.					No. of Complement.	Description and Dilution of cholesterinised Antigen.	Date of Test.
1 : 5	1 : 10	1 : 20	1 : 40	1 : 80			
...	0	0	0	c	59	Ext. 1 (cholest. saturated) 1 : 80 - - -	21.x.18.
...	0	0	0	+++	59	(„ „) 1 : 160 - - -	„
...	...	? 0	+	? c	59	Ext. 3 („ „) 1 : 160 - - -	22.x.18.
...	...	0	0	+++	59	„ (cholest. 0·4 per cent.) 1 : 160 - -	„
...	...	0	0	++	59	Ext. 2 („ „) „ - - -	„
...	...	0	? tr	...	59	Ext. 1 (cholest. saturated) 1 : 160 - - -	„
0	0	0	tr	? c	59	Ext. 3 (cholest. 0·4 per cent.) 1 : 160 - - -	12.xi.18.
0	0	0	+	++++	59	„ („ „) 1 : 40 - - -	„
...	tr	++	c	c	90	Ext. 1 (cholest. saturated) 1 : 160 - - -	25.xi.18.
...	tr	++	? c	c	90	Ext. 3 (cholest. 0·4 per cent.) 1 : 160 - -	„
...	0	+	c	c	59	„ („ „) 1 : 40 - - -	9.xii.18.
...	tr	++++	c	c	59	„ („ „) 1 : 160 - -	„
...	tr	++	c	c	59	Ext. 1 (cholest. saturated) 1 : 160 - -	„
tr	+	++++	c	c	90	Ext. 3 (cholest. 0·4 per cent.) 1 : 40 - - -	14.i.19.
*0	tr	++++	? c	c	90	„ („ „) „ - - -	„*
0	0	+	? c	c	90	„ („ „) 1 : 160 - -	„
? 0	? tr	++++	c	c	90	Ext. 1 („ „) 1 : 40 - -	„
? tr	+	? c	c	...	30	Ext. 3 („ „) „ - - -	15.i.19.
tr	...	c	c	...	33	„ („ „) „ - - -	„
? tr	...	c	c	...	50	„ („ „) „ - - -	„
tr	+	? c	? c	...	52	„ („ „) „ - - -	„
? 0	tr	+++	c	c	64	„ („ „) 1 : 160 - -	21 i.19.
0	tr	++++	c	c	64	„ („ „) 1 : 40 - - -	„
*0	tr	++++	c	c	64	„ („ „) „ - - -	„*
? 0	+	? c	c	c	39	Noguchi Ext. (cholest. 0·5 per cent.) 1 : 640 -	14.iii.19.
? tr	++	c	c	c	59	„ („ „) „ -	„
? tr	tr	+++	? c	c	39	Ext. 3 (cholest. 0·4 per cent.) 1 : 160 - -	„
0	++++	+++	c	c	59	„ „ „) „ - - -	„
c	c	c	...	...	50	Noguchi Ext. (cholest. 0·5 per cent.) 1 : 640 -	31.vii 19.

* Duplicate, except that the antigen was made up so as to be of maximum turbidity.

TABLE XIV.

REPEATED WASSERMANN TESTS upon a strongly POSITIVE SERUM which, after INACTIVATION at 56° C. for half an hour, had been kept STERILE at ROOM TEMPERATUTE in sealed ampoules. (Ice-chest Method.)

Titration of Positive Serum No. 5, bled 21.x.18.						No. of Complement.	Description and Dilution of cholesterinised Antigen.	Date of Test.
1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320			
0	0	0	0	c	c	59	Ext. 1 (cholest. saturated) 1 : 80	21.x.18.
0	0	0	0	++++	c	59	„ („ „) 1 : 160	„
0	0	0	tr	? c	c	59	Ext. 3 (cholest. 0·4 per cent.) 1 : 160	22.x.18.
0	0	0	? tr	++++	...	59	Ext. 2 („ „ „) „	„
0	0	0	tr	++++	...	59	Ext. 1 (cholest. saturated) 1 : 160	„
0	0	0	tr	+++	...	65	„ („ „) „	25.x.18.
0	0	0	+	++	...	65	Ext. 3 (cholest. 0·4 per cent.) 1 : 160	„
0	0	0	++	c	c	59	„ („ „ „) „	12.xi.18.
0	0	0	+++	? c	c	59	„ („ „ „) 1 : 40	„
0	0	+	++++	c	c	90	Ext. 1 (cholest. saturated) 1 : 160	25.xi.18.
0	0	++	c	c	c	90	Ext. 3 (cholest. 0·4 per cent.) 1 : 160	„
0	0	tr	? c	c	c	64	„ („ „ „) 1 : 40	5.xii.18.
0	0	? 0	+	c	c	64	„ („ „ „) 1 : 160	„
0	0	tr	++++	c	c	79	„ („ „ „) 1 : 40	„
0	0	? 0	+	++++	c	79	„ („ „ „) 1 : 160	„
0	0	tr	? c	c	c	57	„ („ „ „) „	6.xii.18.
0	0	+	? c	c	...	76	„ („ „ „) „	„
0	? 0	? c	c	c	...	59	„ („ „ „) 1 : 40	9.xii.18.
0	0	++	c	c	...	59	„ („ „ „) 1 : 160	9.xii.18.
0	0	+	c	c	...	59	Ext. 1 (cholest. saturated) 1 : 160	„
*0	0	++	c	...	...	90	Ext. 3 (cholest. 0·4 per cent.) 1 : 40	14.i.19.
0	0	? c	c	...	...	90	„ („ „ „) „	„
0	0	++	c	...	...	90	„ („ „ „) 1 : 160	„

TABLE XIV.—*continued.*

Titration of Positive Serum No. 5, bled 21.x.18.						No. of Complement.	Description and Dilution of cholesterinised Antigen.	Date of Test.
1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320			
0	tr	c	c	...	...	90	Ext. 1 (cholest. 0·4 per cent.) 1 : 40 - - -	14.i,19.
0	0	+	++++	...	...	30	Ext. 3 (,, ,, ,,) ,, - - -	15.i.19.
0	? tr	++++	? c	...	...	33	,, (,, ,, ,,) ,, - - -	,,
0	tr	++++	c	...	...	50	,, (,, ,, ,,) ,, - - -	,,
0	tr	+++	c	...	...	52	,, (,, ,, ,,) ,, - - -	,,
*0	tr	++	c	...	...	30	,, (,, ,, ,,) ,, - - -	17.i.19.
0	+	? c	c	...	...	30	,, (,, ,, ,,) ,, - - -	,,
0	tr	++	c	...	...	30	,, (,, ,, ,,) 1 : 160 - -	,,
0	tr	+++	? c	c	...	64	,, (,, ,, ,,) ,, - - -	21.i.19.
*0	tr	++++	c	c	...	64	,, (,, ,, ,,) 1 : 40 - - -	,,
0	tr	? c	c	c	...	64	,, (,, ,, ,,) ,, - - -	,,
? tr	+	++	++	++	...	36	,, (,, ,, ,,) ,, - - -	23.i.19.
0	? 0	tr	+	+	...	39	,, (,, ,, ,,) ,, - - -	,,
0	tr	+++	c	c	...	83	,, (,, ,, ,,) ,, - - -	,,
0	tr	+	+++	++	...	Mixed	,, (,, ,, ,,) ,, - - -	,,
? tr	+	++	+++	++++	...	36	,, (,, ,, ,,) 1 : 160 - -	,,
0	0	tr	+	+	...	39	,, (,, ,, ,,) ,, - - -	,,
0	tr	++	c	c	...	83	,, (,, ,, ,,) ,, - - -	,,
0	tr	++	++++	++++	...	36	Ext. 1 (,, ,, ,,) 1 : 40 - - -	,,
0	0	? tr	+	+	...	39	,, (,, ,, ,,) ,, - - -	,,
0	0	+	c	c	...	83	,, (,, ,, ,,) ,, - - -	,,
0	? tr	+	++++	? c	...	Mixed	,, (,, ,, ,,) ,, - - -	,,
0	tr	++++	c	...	...	39	Noguchi Ext. (cholest. 0·5 per cent.) 1 : 640 -	14.iii.19.
0	tr	? c	c	...	...	59	,, ,, (,, ,, ,, ,, -	,,
0	? tr	++	++++	...	...	39	Ext. 3 (cholest. 0·4 per cent.) 1 : 160 - -	,,
0	0	++	c	...	...	59	,, (,, ,, ,, ,, - -	,,
tr	++	c	c	...	...	50	Noguchi Ext. (cholest. 0·5 per cent. 1 : 640) -	31.vii.19.

* Duplicate of the following test except that the antigen dilution was prepared so as to give maximum turbidity.

TABLE XV.

Repeated Wassermann Tests upon a Positive Serum which, after Inactivation at 56° C. for half an hour, had been kept Sterile at Room Temperature in sealed ampoules. (Ice-chest Method.)

Titration of Positive Serum No. 6, bled 21.x.18.					No. of Complement.	Description and Dilution of cholesterinised Antigen.	Date of Test.
1 : 10	1 : 20	1 : 40	1 : 80	1 : 160			
0	0	0	tr	c	59	Ext. 1 (cholest. saturated) 1 : 80	21.x.18.
0	0	0	tr	c	59	„ („ „) 1 : 160	„
0	0	? tr	+++	c	59	Ext. 3 („ „) „	22.x.18.
0	0	0	tr	? c	59	Ext. 3 (cholest. 0·4 per cent.) 1 : 160	„
0	0	? tr	++++	...	59	Ext. 2 („ „ „) „	„
0	0	0	+	c	59	Ext. 1 (cholest. saturated) 1 : 160	„
0	0	? tr	++	++++	65	„ („ „) „	25.x.18.
0	0	0	tr	+	65	Ext. 3 (cholest. 0·4 per cent.) 1 : 160	„
0	0	0	++	c	59	„ („ „ „) „	12.xi.18.
0	0	0	++	? c	59	„ („ „ „) 1 : 40	„
0	tr	+++	c	c	90	Ext. 1) cholest. saturated) 1 : 160	25.xi.18.
0	tr	+++	c	c	90	Ext. 3) cholest. 0·4 per cent.) 1 : 160	„
0	tr	? c	c	c	59	„ („ „ „) 1 : 40	9.xii.18.
0	tr	++++	? c	c	59	„ („ „ „) 1 : 160	„
0	tr	? c	c	c	59	Ext. 1 (cholest. saturated) 1 : 160	„
*0	tr	++++	c	...	90	Ext. 3 (cholest. 0·4 per cent.) 1 : 40	14.i.19.
0	tr	? c	c	...	90	„ („ „ „) „	„
0	tr	++	c	...	90	„ („ „ „) 1 : 160	„
0	tr	c	c	...	90	Ext. 1 („ „ „) 1 : 40	„
0	tr	+	c	...	30	Ext. 3 („ „ „) „	15.i.19.
? 0	+	? c	c	...	33	„ („ „ „) „	„
? tr	+	++++	c	...	50	„ („ „ „) „	„
? 0	tr	++++	? c	...	52	„ („ „ „) „	„
0	+	++++	? c	...	64	„ („ „ „) 1 : 160	21.i.19.
*0	tr	c	c	...	64	„ („ „ „) 1 : 40	„
0	+	? c	c	...	64	„ („ „ „) „	„

TABLE XV.—*continued.*

Titration of Positive Serum No. 6, bled 21.x.18.					No. of Complement.	Description and Dilution of cholesterinised Antigen.	Date of Test.
1 : 10	1 : 20	1 : 40	1 : 80	1 : 160			
0	tr	++	? c	...	39	Noguchi Ext. (cholest. 0·5 per cent.) 1 : 640 -	14.iii.19.
0	+	? c	c	...	59	„ („ „ „) „ -	„
0	? 0	+	+++	...	39	Ext. 3 (cholest. 0·4 per cent.) 1 : 160 - -	„
0	? tr	+++	c	...	59	„ („ „ „) „ - - -	„
0	+	c	c	...	59	Ox Ext. cholest. (0·016 per cent.) 1 : 160 - -	„
+	++++	c	c	...	50	Noguchi Ext. (cholest. 0·5 per cent.) 1 : 640 -	31.vii.19.

* Duplicate of the following test, except that the antigen was made up so as to be of maximum turbidity.

While their main function is to illustrate the degree of persistence of the Wassermann reacting property in stored syphilitic sera, Tables XIII., XIV., and XV. record, in addition, a number of other observations. It will be noted that, on each date on which the sera were re-tested, more than one test was made. The duplicates vary on different occasions but include comparisons :—

(1) between the results obtained with different specimens of complement (other factors being the same) ;
(2) between those obtained with different heart extracts and with heart extracts containing different proportions of cholesterin ;
(3) between those obtained with different dilutions of the same antigen suspension ; and
(4) between those obtained with the same dilution of the same antigen but with suspensions made up so as to differ in turbidity (rapid or slow mixing).

For example, on 15.i.19 on each of the three tables comparison is made between four complements, other factors in the reactions being constant ; definite slight differences in the titre of each positive serum are evident. The titrations on 23.i.19 of Table XIV. show similar variations. These differences we have already referred to (p. 24) ; we consider them inherent and unavoidable in all complement fixation tests performed with different specimens of guinea-pig's serum.

TABLE XVI.

Comparison of Different Extracts from the same Human Heart.

Preparation:
1 = 10 grams extracted with 25 c.c. Absolute Alcohol - -
2 = „ „ „ „ 50 c.c. „ „ - -
3 = „ „ „ „ 100 c.c. „ „ - -
4 = „ „ „ „ 200 c.c. „ „ - -
B = Extract obtained by treating residue of Extract 2 with 50 c.c. Absolute Alcohol - - - - - -

All for 8 days at 37° C.

Min. = Minimum turbidity obtained by rapid mixture of extract and saline.

Max. = Maximum turbidity obtained by floating extract on surface of saline and slowly mixing.

Wassermann Reaction by Cold Fixation Method.

	Serum.	Extracts 1 : 15 in Saline.										Extracts 3 parts, Cholesterin (1 per cent.) 2 parts, 1 : 100 in Saline.									
		1.		2.		3.		4.		B.		1.		2.		3.		4.		B.	
		Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.
	1 : 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1 : 10	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1 : 20	? c	+	+++	0	? 0	0	0	0	0	0	? tr	0	0	0	0	0	0	0	0	0
	1 : 40	c	? c	c	++++	c	++	? 0	0	+	? 0	c	? 0	+	0	0	0	0	0	0	0
	1 : 80	c	c	c	c	c	c	c	+	c	c	c	++	c	? 0	0	0	0	? tr	0	? tr
		*Titre																			
Complement 1 : 25.	·25 c.c.	c	c									c	c			c	c			c	c
	·2 c.c.	c	c									c	c			c	c			? c	? c
	·15 c.c.	c	c									c	c			? c	? c			++++	++++
	·1 c.c.	++++	? c									+++	+++			++	++			+	+

* These readings represent the hæmolytic activity of the complement kept in contact with saline only ; the corresponding readings in columns to the right represent the effect on this of contact with the antigen indicated at the top of each column.

TABLE XVI.—*continued.*

COMPOSITION OF EXTRACTS.

	I.	II.	III.	IV.	V.	VI.
Extract.	Dry Residue in grams per 100 c.c.	Weight after Acetone Extraction in grams per 100 c.c.	Weight Acetone soluble in grams per 100 c.c.	Proportion of Acetone soluble in Dry Residue.	Dry Residue from Extracts saturated with Cholesterin in grams per 100 c.c.	Percentage Cholesterin dissolved.
				Per cent.		
1	1·908	? (impossible to dry).	0·454	23·7	—	—
2	1·110	0·744	0·316	28·7	1·336	0·226
3	0·580	0·360	0·220	38·0	1·102	0·522
4	0·308	0·148	0·160	52·0	1·084	0·776
B	0·430	0·098	0·328	76·3	1·450	1·020
H.I.	0·715	—	—	—	1·040	0·325
H.II.	0·285	—	—	—	1·345	1·060

ANTIGEN.

The active principle of the Wassermann antigen, it is generally agreed, is contained in the lipoidal substances obtained by extracting normal organs with absolute alcohol. These substances are divided into two main groups, according as they are soluble or insoluble in acetone. In this section we give our reasons for recommending the employment of an alcoholic solution of acetone-insoluble lipoids with added cholesterin.

(1) *Acetone-soluble and Acetone-insoluble Fractions.*—In an ordinary qualitative Wassermann test simple alcoholic extracts made from different hearts of the same species (human, guinea-pig or ox) give fairly uniform results, provided that the conditions of extraction are identical. The hearts should be used at approximately the same period after death and the ratio of heart muscle to alcohol in the extraction process must be the same.

This ratio has a profound influence upon the composition of the resulting extract and upon its action in the Wassermann reaction. Table XVI. is an illustration. Five extracts from the same heart made with different quantities of alcohol were compared in regard to their composition and action upon complement in the presence of graded quantities of syphilitic serum. The result of the test shows that the greater the proportion of alcohol used to extract the heart muscle, at least up to the ratio of 20 of the former to 1 of the latter, the stronger is the antigenic effect

of the resulting extract, and this whether used alone or with added cholesterin. Further, the second extract from the same portion of heart muscle acts more strongly than the first (*cf.* Extract 2 and Extract B). The latter observation has been made in the case of extracts from each of nine different hearts and appears to be the rule.

These differences in the potency of the extracts can be correlated with differences in the proportions to each other of the acetone-soluble and acetone-insoluble substances which they contain. These proportions are shown in the second part of Table XV. They were ascertained in the following manner. In each case 5 c.c. of extract was evaporated to dryness *in vacuo* over sulphuric acid and the residue was weighed (column I). The dry residue was then extracted with acetone and again dried and weighed (column II); the acetone extract was also evaporated and the deposit was dried and weighed (column III.).

It will be observed that the ratio of acetone-soluble to acetone-insoluble substances in the extracts rises as the volume of alcohol relative to heart muscle increases and that the second extract (B) contains the highest proportion (column IV). This increase is, no doubt, chiefly due to the fact that the higher proportions of alcohol are less diluted with water from the extracted tissue and consequently dissolve out more readily the cholesterin which forms the main component of the acetone-soluble fraction of the lipoids. Direct evidence of this is shown in columns V. and VI., which represent the dissolving power of the different extracts for added cholesterin; cholesterin crystals were shaken up at 37° C. in the original alcoholic extracts and the solutions allowed to crystallise out at room temperature: after settling, the supernatant fluid was evaporated to dryness and the deposit weighed: the increase over the original dry weight is taken to be due to dissolved cholesterin. For comparison it may be mentioned that alcohol containing 5 per cent. of water will dissolve under the same conditions 0·85 per cent. cholesterin, with 10 per cent. of water 0·65 per cent. cholesterin, with 20 per cent. water 0·2 per cent. cholesterin, and with 40 per cent. water only 0·06 per cent. cholesterin.

To sum up, then, it appears that the content of acetone-soluble substances increases in proportion to the amount of alcohol used to extract a similar quantity of heart muscle, and that this increase in acetone-soluble substances corresponds to an increase in capacity to act as antigen in a W.R. Since cholesterin is probably the most important acetone-soluble substance, its influence as a component of antigen requires discussion.

(2) *Influence of Cholesterin in the Wassermann Reaction.*—It has become a common practice to reinforce the alcoholic extract of normal heart by the addition of cholesterin, and there

is no doubt that such cholesterinised extracts give far more positive results than the simple extracts. This increased delicacy is obtained at the expense of some diminution of the safety of the test, slight though it may be. The ideal antigen should have no effect upon complement except in the presence of syphilitic serum. But it has been shown in the section on complement that the addition of cholesterin to a simple extract, which alone had no effect on a particular complement, made it strongly anticomplementary (*vide* Table IV., complement 76). It is true that the complement was exceptional, but weaker manifestations of the same phenomenon occur with many specimens of complement and the observation indicates the necessity for caution in the use, in a complement fixation test, of an antigen which has such potentiality. Furthermore, we have found that some cholesterinised extracts do not always give frank negatives with non-syphilitic sera after due precautions have been taken to allow for the anticomplementary action of the antigen alone. In a series of 209 patients suffering from non-syphilitic skin diseases, a mixture of five different heart extracts to which 1 per cent. cholesterin had been added (in the proportion of 2 to 3 parts of extract, used in a dilution of 1 in 20), gave with the ordinary technique (fixation at 37° for 1 hour) definite positive reactions in the case of four persons in whom syphilis was highly improbable, and in 35 similar cases failed to give frank negatives; in 17 of these lysis of less than half the cells occurred. The same antigen used highly diluted (1 in 160) with contact at ice-chest temperature overnight produced a still higher proportion of doubtful reactions. The explanation is probably that the proportion of cholesterin to extract was excessive. Sachs has shown that different extracts require different amounts of cholesterin. Our observations given above upon the proportion of acetone-soluble and insoluble substances in different extracts show that, apart from inherent differences in composition of the heart muscle, the method of extraction affects the ratio which would be required.

(3) *Mode of Action of Cholesterin.*—The probable mechanism of its action in the test we conceive to be as follows :— The acetone-insoluble lipoids form in saline a relatively stable colloidal solution, whereas cholesterin in similar circumstances flocculates out at once. The mixture of the two, however, perhaps in virtue of a loose combination, forms a clear opalescent or faintly turbid colloidal solution, the degree of clarity and the stability of which are in proportion to (1) the relative predominance of acetone-insoluble lipoids over cholesterin, and (2) the rapidity with which the transference from true alcoholic solution to colloidal solution in saline is effected. When the interaction takes place between the extract lipoids and the Wassermann substance in the serum, the cholesterin is precipitated in the form of fine particles, and this precipitate in the nascent state acts as a powerful adsorber

of complement. When the cholesterin is large in amount in proportion to the lipoids, the slightest interaction between the latter and the Wassermann substance will result in an abundant discharge of cholesterin and the production of a strongly positive reaction. On the other hand, such heavily cholesterinised extracts, probably already precipitating spontaneously, appear to act independently upon the more unstable globulins such as are present in "sensitive" guinea-pig serum and in certain non-syphilitic human sera. On the latter hypothesis one might explain the false positive and doubtful results which have been referred to above.

The rôle of cholesterin in the Wassermann reaction may be likened to the action of an intensifier in photography which renders printable the image of an under-exposed negative. The cholesterin similarly intensifies the action on complement of the complex formed by the extract lipoids and the Wassermann substance, and enables a weak interaction to be demonstrated by increasing the amount of complement inhibited, not by increasing the capacity of organ extract to react with syphilitic antibody.

Thus, if a positive serum is tested by the method of dilution to extinction of its reacting power, it is found that the positive reaction with extract alone can often be demonstrated with the same high dilution of serum as with the same extract plus added cholesterin, provided that the amount of complement used is sufficiently small (*see* Table XVII., p. 55). The experiment recorded in this Table is one of several similar, but cannot be reproduced with all syphilitic sera and all complements.

(4) *The Acetone-insoluble Lipoid Fraction.*—The preceding observations have indicated that, while increase in the acetone-soluble content of an extract—including increase produced by the artificial addition of cholesterin—increases its efficacy in the complement fixation test, there is a danger that excess of this component may exercise a non-specific anticomplementary action and thereby create "false positives."

We may now consider the possible advantage of employing separately as antigen the acetone-insoluble lipoid fraction as proposed by Noguchi.

For convenience we quote here the description by Craig* of the preparation of Noguchi extract:—

> "Normal organs are used and may be from man or the lower animals. Extracts made from heart muscle, liver, or kidney of man, or the same organs of the guinea-pig or rabbit, are satisfactory, and the extraction is done in the same manner as for the antigens already described, 10 parts of absolute alcohol being used for each part of

* Craig, *The Wassermann Test*, Kimpton, London, 1918.

tissue. After extraction, the material is filtered, and the filtrate evaporated to dryness by the aid of an electric fan. The residue is taken up with ether, and the solution thus obtained is allowed to stand overnight in a cool place. In the morning the supernatant ether will be found perfectly clear, while the insoluble portion of the residue has settled to the bottom of the receptacle. The clear ethereal portion is decanted off and evaporated to a small quantity and mixed with 10 volumes of pure acetone. The precipitate which forms is allowed to settle, and the supernatant liquid poured off. The precipitate is light brown in colour, and forms the material from which the antigen [suspension] is made, 0·3 gram of this acetone-insoluble fraction being dissolved in 1 c c. of ether and then mixed with 9 c.c. of methyl alcohol."

We have found that ox heart forms an equally satisfactory source of acetone-insoluble lipoid prepared as above. We have not yet sufficient data to determine the limits within which preparations from different ox hearts may vary.

It has been shown by Noguchi that the acetone-insoluble fraction of the alcoholic extract contains the essential constituents of the Wassermann antigen. Moreover, treatment with acetone removes various undesirable substances, such as soaps and fats, and the final product is less anticomplementary than many crude alcoholic extracts. This attempt by Noguchi to obtain the antigenic substances in a purer state is an important step and is a subject worthy of further attention by bio-chemists.

But the Noguchi extract alone sometimes fails to detect syphilitic sera which give a definite positive reaction with simple alcoholic heart extracts, with or without cholesterin. It is evident that, to obtain the most delicate reactions, some proportion of the acetone-soluble lipoid (cholesterin) must be present in addition. Is it possible to combine the adjuvant action of the latter with the pure specific action of the former?

(5) *Standardisation of Antigen.*—If our conception of the mode of action of cholesterin is correct, it should be possible to adjust the proportion of cholesterin in the total lipoid contents so that the intensifying action of the former is obtained, while its precipitating effect on the serum globulins, however unstable, is reduced to a minimum. Such an antigen would be both safe and powerful. But in attempting this with crude organ-extract one is met with certain difficulties. In the first place such extracts often contain anticomplementary substances which apparently take no part, or at any rate no essential part, in the specific reaction yet complicate this by their independent inhibitory action on complement. Secondly, they vary very much in the amount of cholesterin they contain and some already contain an excessive amount; the latter condition appears especially common in extracts from guinea-pigs' hearts.

It has seemed preferable to us, therefore, to take as the basis of the antigen the acetone-insoluble fraction of the crude organ-extract as first suggested by Noguchi. This is of more uniform composition as obtained from different hearts and can be freed from anticomplementary impurities. Cholesterin of definite purity and in a uniform state can then be added; in this way one can prepare a series of antigens in which the ratio of acetone-soluble to acetone-insoluble lipoid is accurately known, and can then determine by experiment the ratio which gives the nearest approximation to the ideal antigen. The experiment recorded in Tables XVIII. and XIX. (pp. 56–57), has been arranged with this object: these tables must be read together, each forming part of the same experiment.

Different amounts of acetone-insoluble extract (Noguchi) were mixed with 1 per cent. alcoholic solution of cholesterin. These mixtures were diluted with saline so as to give a colloidal solution of minimum turbidity (rapid mixture), and the effects of the various dilutions were tested (1) upon a sensitive and an insensitive complement alone, *i.e.*, in the presence of salt solution (lower part of Table), and (2) (upper part of Table) in a Wassermann test with different dilutions of a positive serum together with a fixed amount of each of the above-mentioned complements (in parallel experiments).

A positive serum of moderate strength was chosen, and a number of sets of dilutions were made ranging from 1 : 40—1 : 640. Mixtures of extract and 1 per cent. cholesterin were made, beginning with 2 parts of extract to 1 part of 1 per cent. cholesterin up to 0·5 of the former and 1 of the latter. From these mixtures dilutions with salt solution ranging from 1 : 160 to 1 : 1280 were prepared. Two fresh complements were selected, one (No. 77) sensitive to cholesterinised antigen, the other (No. 84) insensitive. The two complements were titrated alone and with each antigen mixture diluted from 1 : 160—1 : 1280. The sets of serum dilutions were mixed with 1 vol. of each antigen dilution and with 1 vol. of each complement diluted 1 : 25. All the tubes were placed in the ice-chest for 16 hours before the addition of the sensitised cells. In the examination of the results it was noted which mixture of extract and cholesterin just affected the hæmolytic value of the sensitive complement. This was found to be the mixture containing 1 part of extract to 1 part of 1 per cent. cholesterin in a dilution of 1 : 640, and also the 1·5 to 1 in a dilution of 1 in 1280. On referring to the test above with the positive serum it will be seen that the mixture 1—1 in a dilution of 1 in 640 gives a better result than the 1·5—1 in a dilution of 1 in 1280. Therefore the former is chosen as containing the optimum proportions of extract and cholesterin. That is to say, it is not the mixture which will give the strongest reaction with a high dilution of a positive serum, but the one which, while giving a good positive result, will not have an unsafe proportion of cholesterin to extract.

A comparison of the test with complement 77 and the one with complement 84, which is not itself influenced by the antigen, shows that with each complement 1 in 640 dilution of the 1—1 mixture gives the same result with the positive serum, *i.e.*, commencing lysis with the 1 in 160 serum dilution.

Such uniformity, however, cannot always be attained. In the experiment recorded in Table XX., four different Noguchi extracts, one prepared from bovine heart and three from human heart, were compared. They were first standardised in the above way in regard to the optimum proportions of cholesterin. The same serum, No. 12, was used throughout for the standardisation, and in the case of each antigen the dilution and proportions of extract and cholesterin chosen as optimal gave an identical result with this serum. Different complements were used in each experiment. Each antigen, as thus standardised, was then tested with a number of sera, including No. 12.

On the whole these results with each serum and the different antigens were fairly even, that is, partial lysis generally occurred in the same dilution of each serum. But even with serum No. 12, with which the antigens were originally standardised, the results were by no means identical. And with the other sera it was not always the same antigen which gave the best result. It is possible that the divergent results may depend on differences in the colloidal state of the antigen dilutions, since it seems impossible to obtain these in exactly the same condition.

In spite of these differences, due, as suggested above, to differences not in the composition but in the physical state of the antigen suspensions, the standardised antigen gave definitely more uniform results than the crude extract.

In an experiment (Table XXI.) 10 positive sera were tested with the standardised antigen and with the crude extract + 0·4 per cent. cholesterin; two complements were used, one, No. 39, sensitive to a cholesterinised antigen, the other, No. 59, almost insensitive. All the sera gave nearly equal results with the two complements when the standardised extract was used. In the case of the crude cholesterinised extract there was rather more variation in the inhibition of the two complements and the insensitive complement gave generally the stronger results, *i.e.*, the greater fixation of complement.

It will be observed on comparing the crude extract with the standardised extract that the former gave the stronger results, except with the weakest sera. It would be easy to raise the strength of the standardised antigen, but it was designedly made less powerful in action with highly diluted positive sera than the cholesterinised crude extract, since it was observed that the latter, even when highly diluted, did occasionally give non-specific inhibition of complement by the method of prolonged ice-chest fixation.

TABLE XVII.

EXPERIMENT on the ROLE OF CHOLESTERIN, showing that the EXTRACT without added CHOLESTERIN may be as sensitive a REAGENT for detecting the presence of REACTING SUBSTANCE in highly diluted SYPHILITIC SERUM as is the CHOLESTERINISED EXTRACT.

Fixation for 18 hours in the ice-chest.

Serum (Syphilitic) Dilutions as under, 0·25 c.c.	Extract 1 : 40, 0·25 c.c. Complement Dilutions as under, 0·25 c.c.					Extract (containing 0·4 per cent. Cholesterin) 1 : 40, 0·25 c.c. Complement Dilutions as under, 0·25 c.c.				
	1 : 25	1 : 30	1 : 40	1 : 50	1 : 60	1 : 25	1 : 30	1 : 40	1 : 50	1 : 60
1 : 40 - - -	0	0	0	0	0	0	0	0	0	0
1 : 80 - - -	? tr	0	0	0	0	0	0	0	0	0
1 : 160 - - -	++++	++	tr	0	0	0	? tr	0	0	0
1 : 320 - - -	c	c	? ? c	++++	++	c	? c	++	+	+
1 : 640 - - -	c	c	c	? ? c	++++	c	c	c	c	++++
None - - -	c	c	c	c	? c	c	c	c	c	++++

In 1 : 320 dilution of serum the reacting substances are in weak concentration, and the activity of the complement is barely affected. In 1 : 160 dilution of serum the reacting substances are detected by both antigens, but more complement is inhibited in the presence of the serum by the extract + cholesterin than by the extract alone.

TABLE XVIII.

METHOD of ascertaining the OPTIMUM PROPORTION of 1 per cent. CHOLESTERIN to be added to NOGUCHI ACETONE-INSOLUBLE EXTRACT to obtain a safe ANTIGEN for ICE-CHEST FIXATION.

Complement Fixation in the Ice-chest overnight.

Proportion of Extract to 1 per cent. Cholesterin.	1 vol. of 1 : 25 Complement No. 77 (a "Sensitive" Complement). 1 vol. of each Antigen Dilution. 1 vol. of No. 12 (Positive) Serum, diluted as under.																			
	40	80	160	320	640	40	80	160	320	640	40	80	160	320	640	40	80	160	320	640
2 — 1	0	+	? c	c	c	0	tr	++	++++	? c	0	tr	+	+++	++++	0	tr	+	++	++++
1·5 — 1	0	tr	++	? c	c	0	? 0	tr	+	++	0	? 0	? tr	tr	tr	0	tr	tr	tr	+
1 — 1	0	? tr	++	+++	? c	0	? 0	tr	+	+++	0	0	tr	tr	+	0	? tr	tr	tr	+
0·8 — 1	0	0	tr	+++	++++	0	0	? tr	tr	tr	0	0	? 0	? 0	? tr	0	? 0	? 0	? 0	? tr
0·5 — 1	0	0	tr	+	+	0	0	? 0	? tr	tr	0	0	? 0	? 0	? tr	+	+++	++++	+	+
Antigen, diluted—	1 : 160					1·320					1 : 640					1 : 1280				
	25	30	40	60		25	30	40	60		25	30	40	60		25	30	40	60	
2 — 1	c	c	? c	++++		c	c	? c	+++		...	...	...	...		...	...	...	...	
1·5 — 1	c	c	? c	+++		c	c	? c	+++		c	c	? c	++		c	? c	+++	++	
1 — 1	c	? c	? c	+++		c	c	? c	+++		c	? c	+++	++		c	? c	++++	+	
0·8 — 1	c	? c	? c	++		+++	++	++++	+		++	+++	+	+		+++	++	+	+	
0·5 — 1	++	+++	++	tr		+++	++	++	tr		++	++	tr	? tr		...	...	+	tr	

1 vol. of Complement 77 diluted as above.
1 vol. of each Antigen Dilution.
1 vol. of Salt Solution.

TABLE XIX.

Proportion of Extract to 1 per cent. Cholesterin.	1 vol. of 1 : 25 Complement No. 84 (an insensitive Complement). 1 vol. of each Antigen Dilution. 1 vol. of No. 12 (Positive) Serum diluted as under.																			
	0	80	160	320	640	40	80	160	320	640	40	80	160	320	640	40	80	160	320	640
2 — 1	0	+	c	c	c	0	tr	? c	c	c	0	? tr	++	? c	c	0	? tr	+	? c	c
1·5 — 1	0	tr	c	c	c	0	? tr	++	c	c	0	? 0	tr	+++	c	0	? tr	tr	+	? c
1 — 1	0	tr	c	c	c	0	0	+	c	c	0	0	tr	++++	c	0	0	tr	++	c
0·8 — 1	0	(	+++	c	c	0	0	tr	++++	c	0	0	tr	+	? c	0	0	? tr	tr	++
0·5 — 1	0	0	tr	? c	c	0	0	? tr	+	c	0	0	? tr	+	+++	0	? 0	tr	+	+
Antigen, diluted—	1 : 60					1 : 320					1 : 640					1 : 1280				
	25	30	40	60		25	30	40	60		25	30	40	60		25	30	40	60	
2 — 1	c	c	? c	++++		c	c	? c	++++		c	c	? c	++++		c	c	? c	++++	

1 vol. of Complement No. 84 diluted as above.
* 1 vol. of each Antigen Dilution.
1 vol. of Salt Solution.

——	Complement Titration.			
	1 : 25	1 : 30	1 : 40	1 : 50
Complement No. 77	c	c	? c	++++
„ No. 84	c	c	? c	++++

Serum 12 control : the 1·5 dilution gave not quite complete lysis with 1 vol. of 1 : 25 complement.

* None of the cholesterinised extracts had the least effect on this complement (No. 84) ; the titres with and without antigen were identical.

TABLE XX.

COMPARISON of FOUR NOGUCHI ACETONE-INSOLUBLE EXTRACTS with added CHOLESTERIN, the proportion of EXTRACT to 1 per cent. CHOLESTERIN having been adjusted by the method described above.

1 vol. of 1 : 25 Complement.
1 vol. of Antigen Dilution.
1 vol. of each Serum Dilution.

Noguchi Extract : Proportion to 1 per cent. Cholesterin.	Dilution of Antigen.	Serum 7 diluted as under.					Serum 9 diluted as under.				
		10	20	40	80	160	10	20	40	80	160
No. 1. 1 – 1.	1 : 640	0	0	+++	c	c	0	tr	++++	c	c
No. 2. 2·5 – 1.	1 : 320	0	0	+	c	c	0	tr	? c	c	c
No. 3A. 3 – 1.	1 : 640	0	0	tr	c	c	...	...	...	...	...
No. 3B. 3 – 1.	1 : 640	0	0	tr	c	c	0	? 0	++++	c	c

Noguchi Extract : Proportion to 1 per cent. Cholesterin.	Dilution of Antigen.	Serum 11 diluted as under.					Serum 12 diluted as under.				
		5	10	20	40	80	20	40	80	160	320
No. 1. 1 – 1.	1 : 640	+++	c	c	c	c	0	0	? tr	+	? c
No. 2. 2·5 – 1.	1 : 320	? c	c	c	c	c	0	0	+	? c	c
No. 3A. 3 – 1.	1 : 640	+	? c	c	c	e	0	0	++++	c	c
No. 3B. 3 – 1.	1 : 640	+	? c	c	c	c	0	0	? c	c	c

Noguchi Extract : Proportion to 1 per cent. Cholesterin.	Dilution of Antigen.	Serum 13 diluted as under.					Serum 14 diluted as under.				
		10	20	40	80	160	20	40	80	160	320
No. 1. 1–1.	1 : 640	0	tr	++++	c	c	0	0	tr	++++	c
No. 2. 2·5–1.	1 : 320	0	+	c	c	c	0	0	+++	c	c
No. 3A. 3–1.	1 : 640	? 0	+++	c	c	c	0	? tr	? c	c	c
No. 3B. 3–1.	1 : 640	? 0	++++	? c	c	c	0	? 0	c	c	c

Noguchi Extract : proportion to 1 per cent. Cholesterin.	Dilution of Antigen.	Serum 15 diluted as under.					Serum 16 diluted as under.				
		10	20	40	80	160	10	20	40	80	160
No. 1. 1 – 1.	1 : 640	0	0	+	? c	c	0	tr	c	c	c
No. 2. 2·5 – 1.	1 : 320	0	0	c	c	c	0	tr	c	c	c
No. 3A. 3 – 1.	1 : 640	0	? tr	c	c	c	0	? 0	? c	c	c
No. 3B. 3 – 1.	1 : 640	0	0	c	c	c	0	0	? c	c	c

None of the antigens had any effect on the titre of the complement (1 : 60 dilution ? c).

All the antigen dilutions were clear, *i.e.*, the salt solution was poured quickly on to the antigen.

TABLE XXI.

COMPARISON of TWO COMPLEMENTS, one (39) SENSITIVE to the ACTION of ANTIGEN alone, the other (59) INSENSITIVE. Experiment shows the Specific Inhibition of each Complement with two Antigens: (A) Noguchi Acetone-insoluble Extract with added Cholesterin (*vide* Table XVIII.), (B) Crude Human Heart Extract (3 parts) and 1 per cent. Cholesterin (2 parts).

No. of Serum.	A. 1 vol. of Noguchi (Chol. 1–1), 1 : 640. 1 vol. of each Serum Dilution as under.									
	1 vol. of 1 : 25 Complement 39.					1 vol. of 1 : 25 Complement 59.				
	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80
1	? 0	+	? c	c	c	? tr	++	c	c	c
2	0	0	tr	++++	? c	0	0	tr	? c	c
3	0	0	tr	+++	c	0	0	tr	++++	c
4	...	0	0	tr	++	...	0	0	tr	++++
5	0	0	tr	++++	c	0	0	tr	? c	c
6	0	0	tr	++	c	0	0	+	? c	c
7	...	0	0	tr	++	...	0	? 0	+++	? c
8	++++	? c	c	c	c	? c	c	c	c	c
9	...	0	0	tr	++	...	0	? tr	++	? c
10	0	tr	+	? c	c	? 0	+	? c	c	c

TABLE XXI.—*continued.*

Serum.	B. 1 vol. of Crude Extract (Chol. 3–2), 1 : 160. 1 vol. of each Serum Dilution as under.									
	1 vol. of 1 : 25 Complement 39.					1 vol. of 1 : 25 Complement 59.				
	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80
1	? tr	tr	+++	? c	c	0	++++	+++	c	c
2	0	0	? 0	++	++++	0	0	0	+	c
3	0	0	? 0	++	++++	0	0	0	+	c
4	...	0	0	? tr	+	...	0	0	0	tr
5	0	0	? tr	++	++++	0	0	0	++	c
6	0	0	? 0	+	+++	0	0	? tr	+++	c
7	...	0	0	0	tr	...	0	0	? 0	tr
8	++++	++++	+++	++	+++	c	c	c	c	c
9	...	0	0	? tr	+	...	0	0	0	0
10	? tr	tr	+	++	++	tr	+	++++	c	c

———	Titration.				Titration + Noguchi. A.				Titration + Crude Extract. B.			
	·25 c.c.	·2	·15	·1	·25 c.c.	·2	·15	·1	·25	·2	·15	·1
1 : 25 complement 39	c	c	+++	+	c	+++	++	tr	+	tr	tr	tr
1 : 25 complement 59	c	c	? c	++	c	c	? c	+++	c	c	++++	++

(6) *Method of Preparation of Antigen Suspensions.*—It is a fact that antigens affect complement differently according as their suspensions are turbid or clear. Generally the more turbid solution is more anticomplementary than the clear, but occasionally the reverse is the case. The globulins of the human sera may react in the same way as those of the guinea-pig to differences in the physical state of the antigen suspension. Tables XXII. and XXIII. represent experiments illustrating the different effects of antigen dilutions similar in composition but differing in physical state. The same fact is shown in the experiment detailed in Table XXIV.

In Table XVI., already referred to (pp. 47–49), the effect of differences in turbidity of the same dilutions of extract (alone and plus cholesterin) is also shown. It would appear from their behaviour towards sensitive complements that these extracts already contained a high proportion of cholesterin. In all cases where the extract alone was used, the antigenic efficiency was greater with the maximum turbidity than with the minimum. With the same extracts plus cholesterin the maximum turbidity again gave greater fixation than the minimum, except in the two (4 and B) where cholesterin was already present in very high proportion in the extracts and with which, as a matter of fact, the suspension, at first of minimum turbidity, was observed soon to become on standing as turbid as the other. This may occur with any antigen, even with rapid mixing, unless the saline is added to the antigen and not *vice versâ*. In the case of these two extracts one may suggest that the method of preparation which gave maximum turbidity caused so much spontaneous discharge of the cholesterin that the intensifying effect was weakened. In practice we recommend rapid mixing, by adding the saline to the antigen, as providing suspensions of greater uniformity from day to day.

The purified heart extract described by Bordet,* and more especially his way of making a suspension from it (*v. infra*), are of particular interest in this connection. Bordet's description came to our notice only after the main body of our report had been written,

We find that Bordet's extract, which resembles the Noguchi extract in being as nearly as possible free from cholesterin, also resembles it in its general behaviour as antigen in the W.R. Like it, when used without added cholesterin and mixed directly with saline, it is a comparatively feeble antigen, but with cholesterin added in proper proportion acts efficiently in high dilution and is not anticomplementary.

Bordet, however, uses it without cholesterin and suspends it in saline in the following manner:—A measured quantity is evaporated to dryness; the lipoid deposit is then rubbed up in a small amount of distilled water and the resulting coarse suspension diluted with normal saline.

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* *Comptes Rendus Soc. Biol.*, 19th July 1919; *B.M.J.*, Vol. II., 1919, p. 353.

TABLE XXII.

Influence of Mode of Preparation of Antigen Suspension upon the Result of a W.R.

Overnight Fixation in the Ice-chest.

(a) *Titration by Multiples of Complement.*

Preparation of Antigen.	1 vol. of Serum 13, 1 in 5. 1 vol. of Ext. 3 (Chol. 0·4 per cent.) 1 : 40. 1 vol. of Complement diluted as under.				
	1 : 8	1 : 6	1 : 5	1 : 4	1 : 3·5
Saline to antigen -	? 0	tr	+	++·++	c
Antigen to saline :—					
(1) Mixed quickly	0	? 0	tr	+	++++
(2) Mixed slowly -	0	tr	++++	c	c

The suspension (antigen to saline (1)) was slightly more anticomplementary than the other two.

(b) *Titration by Dilution of Serum.*

Sera.	1 vol. of 1 : 25 Complement. 1 vol. of each Serum diluted as under. 1 vol. of Extract 3 (Chol. 0·4 per cent.) 1 : 40.									
	Saline to Antigen (clear).					Antigen to Saline (turbid).				
	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320
11 - -	0	? 0	? tr	tr	+	0	+	? c	c	c
12 - -	0	0	0	0	? tr	0	0	0	0	++
13 - -	0	0	? tr	+	++	0	0	tr	c	c
14 - -	0	0	0	tr	+	0	0	0	+	c
15 - -	0	0	tr	+	++	0	0	tr	c	c
16 - -	0	? 0	tr	+	++	0	0	++	c	c

Controls.	1 : 25 Complement Titration.				
	·25 c.c.	·2	·15	·12	·1
Alone - - - -	c	c	c	c	? c
+ clear suspension -	+++	++	+	tr	tr
+ turbid suspension -	c	c	c	c	? c

Antigen diluted	1 vol. of 1 : 25 Complement. 1 vol. of Antigen Ext. 3 (0·4 per cent. Cholest.). 1 vol. of mixed Positive Sera diluted as under.					
	Clear Suspension.			Turbid Suspension.		
	1 : 20	1 : 40	1 : 80	1 : 20	1 : 40	1 : 80
1 : 40 - - -	? tr	++	c	0	0	+
1 : 160 - - -	++	c	c	? 0	+	c

TABLE XXIII.

Influence of Mode of Preparation of Antigen upon the Result of a W.R.

Overnight Ice-chest Fixation.

Preparation of Antigen.	1 vol of Serum 13, 1 : 5. 1 vol. of 1 : 40 Ext. 3 (0·4 per cent. Cholesterin). 1 vol. of Complement diluted as under.				1 vol. of 1 : 25 Complement. 1 vol. of 1 : 40 Ext. 3 (0·4 per cent. Cholesterin). 1 vol. of Serum diluted as under.			
	1 : 8	1 : 6	1 : 5	1 : 4	1 : 20	1 : 40	1 : 80	1 : 160
Saline to antigen	0	? 0	? tr	tr	0	? c	c	c
Antigen to saline :								
Mixed quickly	0	? 0	tr	tr	0	+	c	c
Mixed slowly -	0	0	0	? 0	0	? 0	+	c

The complement was almost insensitive to antigen.

The suspension thus produced we find to be a remarkably efficient antigen ; it equals in efficiency the mixture with cholesterin and, like it, acts best in very high dilution (1 in 640) when employed in the prolonged ice-chest method which we have described.

Noguchi extract (without cholesterin), when prepared in suspension by Bordet's method, has its efficiency similarly enhanced, so that it equals in antigenic potency, at 1 in 640 dilution, the same extract with added cholesterin, but suspended by simple mixing. The Bordet method applied to cholesterinised extract, on the other hand, produces a suspension of diminished instead of improved efficiency.

It is obvious that the addition of cholesterin to the extract is not the only way of producing a lipoid suspension with enhanced power of fixing complement when brought in contact with syphilitic serum ; a similar enhancement may be obtained by the simple process of evaporation and coarse suspension discovered by Bordet. Both methods probably depend for their increased efficiency on some special instability of the reacting lipoids in the suspension.

Our experience with Bordet's antigen prepared in suspension by his method is not sufficient for us to recommend it for routine use in the W.R. to the exclusion of other antigens ; but future routine comparison on a wide scale between cholesterin-free extracts (Bordet and Noguchi) suspended in Bordet's manner and the same extracts cholesterinised as we have described will certainly lead to useful conclusions.

(7) *Optimal proportions of Antigen and Reacting Substances in Serum.*—Table XXIV., which represents an experiment illustrating again the different effects of turbid and clear suspensions of the

TABLE XXIV.

EXPERIMENT showing that the greater FIXING CAPACITY of TURBID SUSPENSIONS of ANTIGEN is less well marked in WEAK than in STRONG CONCENTRATIONS.

Fixation at 37° C. for 1 Hour.

Antigen, Noguchi Extract, 0·5 per cent. Cholesterin.	Turbid Antigen.					Clear Antigen.				
	1 vol. of 1 : 25 Complement. No. 12 Serum diluted as under.									
	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320
1 : 40 - - -	0	? 0	++	...	c	0	tr	++++	? c	c
1 : 80 - - -	0	0	+	c	c	0	? tr	++	? c	? c
1 : 160 - - -	0	0	tr	? c	c	0	0	+	c	c
1 : 320 - - -	0	0	tr	? c	c	0	0	tr	? c	c
1 : 640 - - -	0	0	tr	++++	? c	0	0	tr	? c	c
1 : 1280 - - -	0	0	+	++++	c	0	0	+	? c	c

Complement Controls.

1 : 25 Complement.	Titre of fresh Complement.	Titre after 1 hour at 37°.	+ Turbid Antigen.		+ Clear Antigen.	
			1 : 40	1 : 1280	1 : 40	1 : 1280
0·25 c.c. - - - - -	c	c	? c	c	c	c
0·2 c.c. - - - - -	c	c	++++	? c	? c	+++
0·15 c.c. - - - - -	? c	? tr	+++	? 0	tr	tr
0·1 c.c. - - - -	++	0	...	...	...	...

same antigen, shows further that the stronger concentrations of antigen may be less effective in inhibiting complement than the weaker, at least when in combination with a highly diluted positive serum. For example, the W.R. produced with the 1 in 80 dilution of the syphilitic serum is almost negative (++++ lysis) with 1 in 40 antigen (clear suspension) but much stronger (trace of lysis) with the 1 in 640 dilution of the same suspension.

The same phenomenon is shown incidentally in Table XVIII. on the standardisation of antigen, and in the Tables XIII., XIV., and XV. on the preservation of positive sera. The following experiment (1), recorded in Table XXV., shows the same behaviour with cerebro-spinal fluid.

TABLE XXV.

(1) Experiment illustrating the effect on Complement Fixation of excess of Antigen in proportion to Cerebro-Spinal Fluid (Syphilitic).

Antigen, Noguchi Extract, 0·5 per cent. Cholesterin.	1 vol. of 1 : 25 Complement. 1 vol. Cerebro-spinal Fluid diluted as under. Ice-chest Fixation.					
	Undiluted.	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32
1 : 40 - - -	0	0	? 0	++++	c	? c
1 : 80 - - -	0	0	? tr	tr	? c	c
1 : 160 - - -	0	0	0	? 0	? c	c
1 : 320 - - -	0	0	0	0	+	c
1 : 640 - - -	0	0	0	0	+	c
1 : 1280 - - -	0	0	0	0	+	c
1 : 2560 - - -	0	? 0	? tr	tr	+++	? c
1 : 5120 - - -	tr	+	+	+	? c	? c
1 : 10000 - -	+	+	++++	? c	? c	? c

Complement gave complete lysis in 0·15 c.c. of 1 : 25.
No dilution of antigen had any effect on the titre.

(2) Similar Experiment to above with Inactivated Syphilitic Serum.

Dilutions of Serum 12.	1 vol. of 1 : 25 Complement. 1 vol. of Antigen diluted as under.										
	Noguchi Extract.					Serum 12.	Noguchi Extract + Cholesterin.				
	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1280		1 : 80	1 : 160	1 : 320	1 : 640	1 : 1280
1 : 5	0	0	0	0	0	1 : 40	0	0	0	0	0
1 : 10	0	0	0	0	0	1 : 80	++++	+	? tr	? tr	? tr
1 : 20	? c	++	tr	? 0	0	1 : 160	c	c	c	c	c
1 : 40	c	c	c	c	++++	1 : 320	c	c	c	c	c
1 : 80	c	c	c	c	c	1 : 640	c	c	c	c	c

The 1 : 40 dilution of antigen produced no inhibition with the 1 : 16 dilution of cerebro-spinal fluid, while the 1 : 320, 1 : 640, and 1 : 1280 dilutions of antigen produced marked fixation with the same dilution of fluid. It is evident that there is a considerable latitude in regard to the optimal proportions of antigen and Wassermann reacting substance, since each of the above three dilutions of antigen gave the same amount of fixation. A similar experiment, Table XXV. (2) with a syphilitic serum and cholesterinised and non-cholesterinised antigens showed the same effect. There is no evidence that excess of the reacting substance in the fluid or the serum has the same effect as excess of antigen. When such an effect is apparently obtained with more concentrated serum it must be ascribed to the interference of the serum constituents, as inert colloids, with the adsorption of complement.

It is evident that, if it is desired to demonstrate a minimal quantity of Wassermann reacting substance in a serum, the antigen must be proportionately diluted. To obtain demonstrable complement fixation with such minimal quantities it is necessary that the complement also should be in low concentration. Table XXVI. illustrates this.

A positive result is obtained with the most dilute serum when the antigen is diluted 1 : 160 and the complement 1 : 20 or 1 : 25. If the concentration of complement is increased, the concentration of antigen must be similarly increased, but a positive result will be obtained only with a higher concentration of positive serum. In this experiment two complements were used, one, No. 54, slightly more sensitive to the action of antigen than the other, No. 57 ; it will be observed that the more sensitive complement was less easily fixed. This is most evident with the high concentrations of complement and the low concentration of antigen. It is possible that the serum proteins of the guinea-pig serum which are present in relatively high concentration may interfere with the specific inhibition of the less easily fixed complement. If a still lower concentration of antigen was used, *e.g.*, 1 : 320, a certain amount of inhibition might be obtained with still more dilute serum, but the amount of the reacting substances would be too small to inhibit completely the whole of the volume of 1 in 25 complement.

TABLE XXVI.

Experiment showing the Effect of varying the Concentration of each of the Three Reagents in the Wassermann Test.

Ice-chest Fixation overnight.

Antigen Dilution.	Complement Dilution.	1 vol. of Antigen = Extract saturated in the cold with Cholesterin. 1 vol. Complement 57. 1 vol. of strongly Positive Serum diluted as under.						1 vol. Complement 54. 1 vol. of strongly Positive Serum diluted as under.					
		1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160
1 : 40 Antigen	1 : 25 -	0	0	0	0	+	? c	0	0	0	0	+	+++
	1 : 20 -	0	0	0	0	++	c	0	0	0	0	++	c
	1 : 12·5 -	0	0	0	+	c	c	0	0	0	++	c	c
	1 : 10 -	0	0	0	++	c	c	0	0	? 0	++++	c	c
1 : 80 Antigen	1 : 25 -	0	0	0	0	? 0	++++	0	0	0	0	tr	+++
	1 : 20	0	0	0	0	tr	c	0	0	0	0	tr	++++
	1 : 12·5 -	0	0	0	? 0	c	c	0	0	? 0	+	c	c
	1 : 10 -	0	0	0	tr	c	c	? tr	tr	++	c	c	c
1 : 160 Antigen	1 : 25 -	0	0	0	0	0	++	0	0	0	0	? 0	++
	1 : 20 -	0	0	0	0	0	++++	0	0	0	? 0	+	++++
	1 : 12·5 -	0	0	0	+	c	c	? c	+++	? c	c	c	c
	1 : 10 -	+	+	++	c	c	c	c	c	c	c	c	c

Antigen.	Comp. 57, 1 : 25.	Comp. 54, 1 : 25.
1 : 40	c	++
1 : 80	c	? c
1 : 160	c	? c

Conditions during the Stage of Fixation of Complement.

(*a*) *Temperature of Contact.*—It has been the general custom to conduct the first stage of the Wassermann test at 37° C., or partly at 37° C. and partly at room temperature. But Dean has shown that specific fixation of complement is increased when the constituents of a complement fixation test are mixed and maintained at 0° C., and Smith and MacNeal have proved the value of ice-chest fixation in routine Wassermann work.

Our preliminary experiments have confirmed the experience of others that by means of ice-chest fixation positive results can be obtained in latent cases of syphilis and in treated cases which react negatively in the ordinary method of incubating at 37° C. We have made over 2,000 routine tests in comparison with the laboratory at Rochester Row, and have not found that the warm (37° C.) gives positive results when the ice-chest method fails. Final decision on this point must be reserved, as the series did not include very many primary cases and it has been stated that these may react better by the ordinary warm test than in the cold. Our experiments indicate that the greater effect of cold fixation is not due simply to the period of contact being prolonged. There is little or no increase of specific complement fixation at 37° after a period of one hour. But if, after one hour at 37°, the test is continued at ice-chest temperature overnight, a further increase of complement fixation does occur, although the final amount is not so great as when the tubes are kept at ice-chest temperature throughout. We have therefore come to the conclusion that the method of carrying out the first part of the W.R. in the ice-chest is both reliable and advantageous from the point of view of delicacy.

(*b*) *Order of addition of Ingredients*:—(1) *Effect of preliminary contact of Serum and Antigen before the addition of Complement.*—It has been shown by Dean that if antigen and the appropriate (precipitin) serum are mixed together and kept at 37° C. for a period before the addition of complement, there is less inhibition of complement than when the ingredients are added in rapid succession. A similar result may be obtained with antigen and syphilitic serum, but we have found that this is not invariable, and it is especially difficult to demonstrate if the first part of the experiment is carried out in the ice-chest instead of in the incubator or water-bath at 37°. For example, a positive serum was titrated to its end-point and a number of sets of dilutions were made. After the addition of a 1 in 40 dilution of cholesterinised antigen, the mixed antigen and serum dilutions were placed one set in the water-bath at 37° and the other set in the ice-chest for from 5—60 minutes before the addition of the complement. Opportunity for complement fixation was given by keeping all the mixtures thereafter in the ice-chest for 18 hours. In all cases the results were identical with those obtained when all three ingredients were mixed in rapid

succession in the order—complement, serum, antigen. It was only after a preliminary contact of antigen and serum in the ice-chest for 20 hours that any lessened capacity of the mixtures to inhibit complement was observed.

When, however, the amount of antigen used is minimal, as in the following experiment, Table XXVII., it can be shown that preliminary contact of antigen and serum at 37° may weaken the reaction even though, after the addition of complement, the prolonged ice-chest method of fixation is used.

But if the antigen and serum are mixed together cold, any depreciation of the capacity to inhibit complement as a result of their interaction prior to the addition of the complement is greatly retarded.

Nevertheless we consider it better in all circumstances to avoid bringing the serum and antigen into contact in the absence of complement.

(2) *Effect of preliminary contact of Complement and Antigen before the addition of Serum.*—The effect of antigen upon complement in the medium of salt solution is generally controlled in the test. So long as the contact is not prolonged beyond the period of the control it might be thought that there would be no practical objection to the preliminary mixture and simultaneous addition of these two ingredients. But since the W. test is a method of demonstrating by the action on complement the result of the interaction between antigen and serum, it is preferable that there should be no preliminary contact of the two reagents, antigen and complement, in the absence of serum. Indeed, in the method which we recommend, where a standard amount of complement is used, it is definitely undesirable, even after reduction of the anticomplementary action of the antigen to a minimum. Serum, behaving as a protective colloid, generally preserves complement against the deteriorating action of antigen, but does not restore the lost lytic power once the damage has occurred.

Suggestions on the Theory of the Wassermann Reaction.

(1) *Nature of the Reacting Substance in Syphilitic Serum.*—The hypothesis is generally current that the Wassermann reaction depends upon changes in the degree of precipitability of the serum globulins. Whether this view contains the whole truth or not, it is certain that the precipitation of globulin is an important factor. It may, however, take only a part in the mechanism of the reaction, and may be replaced or reinforced by precipitation of cholesterin. In the case of both the globulin and cholesterin it is possible that their share in the reaction is purely physical. They are the reagents by which the complement is inhibited as a result of their precipitation from colloidal

TABLE XXVII.

Effect of Preliminary Contact of Antigen and Syphilitic Serum on the subsequent Wassermann Reaction.

Extract, + 0·4 per cent. Cholesterin.	Serum dilutions.											
	1 : 20	1 : 40	1 : 80	1 : 160	20	40	80	160	20	40	80	160
1 : 40 - - - -	0	0	? 0	+	0	0	? 0	+	0	0	? 0	+
1 : 80 - - - -	0	0	? 0	+	0	0	? 0	+	0	0	? 0	tr
1 : 160 - - - -	? tr	? tr	+	++	0	0	? 0	+	0	0	? 0	+
1 : 320 - - - -	++++	++++	++++	? c	+	++	++++	++++	+	++	++	+++
	At 37° for 1 hour.				Ice-chest contact for 1 hour.				Antigen, serum, complement in immediate succession cold.			
	Preliminary contact of antigen and serum.											

Note, for example, that the W.R. with the 1 in 80 dilution of syphilitic serum gives a reading of + lysis with 1 in 160 antigen when antigen and serum have been in contact for 1 hour at 37° C. and ? 0 lysis when all the reacting components are mixed at once.

solution. This precipitation is probably secondary to the interaction between two essential substances, the extract lipoids and the Wassermann substance.

That the most readily precipitable globulin, the euglobulin, is not essential to the reaction, is shown by the following experiment, Table XXVIII.

TABLE XXVIII.

Effect on the Wassermann Reaction of precipitating the Euglobulin from a Positive Serum.

The serum (inactivated at 56°) was diluted 1 in 10 with distilled water and was saturated with CO_2 in an ice (water) bath. The deposit was removed by centrifuging and the supernatant fluid was again saturated and centrifuged. Deposit was shaken in salt solution to make 1 in 10 dilution; tonicity was restored to the filtrate by adding the appropriate amount of salt.

Antigen.	1 vol. of 1 : 25 Complement. 1 vol. of each Antigen and each Serum dilution. Fixation, 37° C. for ½ hour.									
	Deposit.			Filtrate.			Whole Serum.			
	1 : 10	1 : 20	1 : 40	1 : 10	1 : 20	1 : 40	1 : 10	1 : 20	1 : 40	
Noguchi, 1 : 40.	? c	c	c	c	c	c	++	c	c	Heated, 60°. Not further heated.
,,	tr	c	c	+	c	c	tr	+	c	

Fixation Ice-chest overnight.

Noguchi, 1 : 640.	c	c	++++	0	+	c	...	...	...	Heated, 60° 10'.
,,	c	c	c	0	+	c	0	tr	? c	Heated, 60° 30'.
,,	? c	c	c	0	+	c	0	? tr	++++	Not further heated.

Removal of the euglobulin from a serum weakens the reaction slightly.

If one compares the results obtained with the whole serum and with the serum after the removal of the euglobulin, it will be seen that (1) the reaction is only slightly weakened, and (2) the reacting substance resists heating to 60° C. for half an hour.

It is well known that heated rabbit serum sometimes gives a positive Wassermann reaction. An attempt was made to determine whether such a reaction could in any way be differentiated from a positive reaction with human syphilitic serum (Table XXIX.).

TABLE XXIX.

WASSERMANN REACTION with INACTIVATED ($\frac{1}{2}$-hour at 56° C.) RABBIT SERA.

Fixation for 1 *hour at* 37° C. *Sera, diluted* 1 : 5. *Antigen* 1 : 40; *turbid suspension of heart extract (with* 0·4 *per cent. cholesterin added).*

Sera.	Quantities of Complement.				
	0·02 c.c.	0·03 c.c.	0·04 c.c.	0·05 c.c.	0·06 c.c.
+ Human, No. 16 - -	0	? 0	++	c	c
Rabbit, 2 - - - -	? 0	+	c	c	c
„ 4 - - - -	c	c	c	c	c
„ 6 - - - -	c	c	c	c	c

Fixation overnight in Ice-chest. Sera, diluted 1 : 5. *Antigen,* 1 : 15; *turbid suspension of heart extract (with* 0·4 *per cent. cholesterin added).*

Sera.	Quantities of Complement.				
	0·02 c.c.	0·03 c.c.	0·04 c.c.	0·05 c.c.	0·06 c.c.
+ Human, No. 16 - -	0	+	++	+++	c
Rabbit, 2 - - - -	0	0	? tr	tr	+
„ 4 - - - -	0	0	? tr	tr	...
„ 6 - - - -	0	? tr	tr	+	...

Sera from three rabbits and one positive human serum were tested at 37° C., and at ice-chest temperature with heart extract plus 1 per cent. alcoholic solution of cholesterin, in the proportion of 3 parts to 2 parts, and with varying amounts of complement. At 37° C. one rabbit serum was positive; at ice-chest temperature all three were positive. In the above tests the antigen was fairly concentrated, *i.e.*, 1 in 40 and 1 in 15. A third experiment was made with the same sera and three additional rabbit sera and two antigens, one a cholesterinised Noguchi extract, the second the same antigen as before. It will be seen that the 1 in 640 dilution of the Noguchi antigen gives slight inhibition with the rabbit sera while the 1 in 40 dilution gives marked inhibition. On comparing the results with the human serum, the 1 in 40 dilution of the Noguchi extract gives only slightly better results than the 1 in 640. The crude extract, which gave a stronger result with the rabbit sera, gives with the human serum a weaker

TABLE XXIX.—*continued.*

Fixation overnight in Ice-chest.

1 vol. of each Antigen Dilution.
1 vol. of 1 : 25 Complement.
1 vol. of each Serum diluted as under.

Antigen.	Rabbit 2.			Rabbit 4.			Rabbit 6.			Rabbits 1, 3, 5.			+ Human Serum 16.					
	1 : 5	1 : 10	1 : 20	1 : 5	1 : 10	1 : 20	1 : 5	1 : 10	1 : 20	1 : 5	1 : 5	1 : 5	1 : 10	20	40	80	160	
Noguchi (0·5 per cent., chol.), 1: 640.	++++	+++	++++	++++	++	++++	++++	+++	c	? c	++++	+	0	tr	c	c	c	Inact at 56° C., ½ hour.
Noguchi (0·5 per cent., chol.), 1: 40.	0	0	? tr	0	0	0	0	0	tr	0	0	? tr	0	? tr	++	c	c	
R.R. ext. (0·4 per cent., chol.), 1:160.	0	0	0	0	0	? 0	0	0	tr	tr	? tr	0	tr	+	? c	c	c	
Noguchi (0·5 per cent., chol.), 1: 640.	? c	c	c	c	c	c	c	c	c	c	c	? c	0	+	c	c	c	Inact. at 60° C., ½ hour
R.R. ext. (0·4 per cent., chol.), 1: 160	+	++	? c	c	c	c	c	c	c	c	c	0	tr	tr	+	c	c	

Controls. Complement gave complete lysis with a 1 : 60 dilution.
Neither sera nor antigen alone inhibited 1 : 25 complement.
Noguchi antigen = acetone-insoluble fraction mixed with equal parts of 1 per cent. cholesterin.
R.R. extract = crude extract of human heart with 1 per cent. cholesterin (3 parts extract to 2 parts cholesterin).

result than the Noguchi extract. Inactivation at 60° C. destroys the reactions with the rabbit sera (with one exception), but effects only a slight reduction in the case of the human serum.

There appears, therefore, to be a difference between the positive Wassermann reaction of rabbit serum and that of human syphilitic serum. The former is strengthened by the use of the more concentrated and highly cholesterinised antigens, and is weakened by exposure to a temperature of 60° C. In the case of the human serum these two factors have much less influence.

This experiment appears to afford some support for the hypothesis that there is in syphilitic serum some relatively thermostable substance, not the globulin, which gives rise to the positive reaction, and that this substance is not present in rabbit serum. The positive reaction in rabbit serum may be due to unstable globulin only, and similar unstable globulin may be the factor which causes the stronger reaction often given by unheated human syphilitic serum, when tested by the ordinary technique, as compared with the same serum inactivated.

(2) *Mechanism of the Reaction.*—One may conceive that there are two processes at work in the Wassermann reaction, one which depends on the presence in the serum of an unknown substance formed through the activities of the spirochaete, the other which is a property of the globulin of the serum. The former, that not exclusively associated with globulin, is the more specific part of the reaction; the latter is found in syphilitic serum, but is not peculiar to it. The former, the "Wassermann substance," has an affinity for the lipoids in the alcoholic organ extract and their interaction results in the formation of a fine precipitate, which, in its nascent condition, has the property of adsorbing, that is, of throwing out of colloidal solution the particles of globulin which are essential to the lytic activity of complement. This capacity can be increased by the addition of cholesterin to the extract lipoids. As has been mentioned, cholesterin seems to form a loose combination with the lipoids of the organ extract; this combination, unlike pure cholesterin, can remain in colloidal solution in saline, but when interaction takes place between the Wassermann substance and the extract, the cholesterin is thrown out. This cholesterin, in the form of a nascent precipitate, adds powerfully to the anticomplementary property of the hypothetical precipitate resulting from the specific interaction of Wassermann substance and extract lipoids.

The reaction may also be assisted by the second of the two processes. This second part depends upon the degree of stability of the serum globulin in the serum to be tested. This globulin, when in the unstable state, is only partially stabilised by inactivation,* and is readily adsorbed or thrown out of

* This may explain the fact mentioned above that a fresh syphilitic serum when tested by the ordinary technique often reacts more strongly than it does after inactivation.

colloidal solution when interaction occurs between Wassermann substance and lipoids; the resulting precipitate in the nascent condition is itself a powerful adsorber of the complement-containing globulin of the guinea-pig serum. In the Wassermann test performed with a simple alcoholic extract alone, the fixation of complement must depend to a greater extent upon this precipitation of syphilitic globulin since there may be comparatively little cholesterin precipitate to act as an adsorbent for complement. The fact that the W.R. with extract alone probably depends on a globulin precipitate does not necessarily imply that non-syphilitic sera containing unstable globulin would be apt to react similarly. The extract alone, poorly provided with cholesterin, may be assumed to act as a precipitant of globulin only after contact with the hypothetical Wassermann substance. As a matter of fact, with rare exceptions, the extract alone does not affect even such very unstable globulins as those of "sensitive" guinea-pig complement.

It can be shown, by experiments in which the proportions of antigen and serum are varied, that in certain circumstances antigen and serum are mutually replaceable as factors in the fixation of complement. For example, when using as antigen an extract containing cholesterin to the amount of 1 per 1,000, the following results were obtained :—

Antigen 1 : 5 dilution	+	serum 1 : 10	}	fixed 0·07 c.c. complement.	
,, 1 : 10	,,	,, ,, 1 : 5			
,, 1 : 10	,,	,, ,, 1 : 20	}	fixed 0·035 c.c. complement.	
,, 1 : 20	,,	,, ,, 1 : 10			

The circumstances in which it is possible to substitute a proportion of one reagent for the same proportion of the other in the fixation of complement seem to depend upon the relative proportions of extract lipoids and added cholesterin in the antigen. For, if crude extract without added cholesterin is used as the antigen, it does not seem to have the same value as the serum, at least in the higher concentrations. This is evident in the following experiment :—

Antigen 1 : 5 dilution	+	serum 1 : 10	fixed 0·06 c.c. of complement.				
,, 1 : 10	,,	,, ,, 1 : 5	,, 0·04 ,, complement.				
,, 1 : 5	,,	,, ,, 1 : 20	,, 0·03 ,, complement.				
,, 1 : 20	,,	,, ,, 1 : 5	,, 0·02 ,, complement.				

If, on the other hand, the antigen is one which is highly cholesterinised, *e.g.*, extract and 1 per cent. cholesterin in equal proportions, dilution of antigen does not diminish fixation so much as dilution of serum.

For example—

Antigen 1 : 5 dilution + serum 1 : 10 fixed 0·07 c.c. of complement.

„ 1 : 10 „ „ „ 1 : 5 „ „ of complement.

„ 1 : 5 „ „ „ 1 : 20 „ 0·03 c.c. of complement.

„ 1 : 20 „ „ „ 1 : 5 „ 0·04 „ of complement.

The behaviour, however, is not the same when a minimal amount of one or other of the two ingredients, antigen or serum, is used. In such cases the less concentrated reagent is able to react with only a certain proportion of the other reagent, no matter what the concentration of the latter may be The experiment recorded in Table XXX. may be quoted.

TABLE XXX.

	1 vol. of each Extract Dilution.									
	1 vol. of 1 : 25 Complement. 1 vol. of Serum 142 diluted as under.					1 vol. of 1 : 10 Complement. 1 vol. of Serum 142 diluted as under.				
Dilution of Extract -	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80
1 : 10	0	0	0	0	tr	0	0	0	tr	++++
1 : 20	0	0	0	0	tr	0	0	tr	++	c
1 : 40	0	0	0	0	tr	+	++	c	c	c
1 : 80	0	0	? 0	tr	++	c	c	c	c	c

Fixation in the ice-chest overnight. 1 vol. of 1 : 40 complement gave complete lysis of cells, alone and in the presence of the extract.

A syphilitic serum was titrated from 1 in 5 to 1 in 80 dilution, and was tested with varying dilutions of extract without added cholesterin : 0·01 c.c. of complement was used. With extract ranging from a dilution of 1 in 10 to 1 in 40, exactly the same amount of lysis, *i.e.*, a trace, was given with the 1 : 80 dilution of serum. But if a test was made with more concentrated serum and complement, it was found that 1 in 20 antigen and 1 in 20 serum inhibited 0·025 c.c. of complement. Halving the amount of serum (1 in 40) and doubling the amount of extract (1 in 10) produced a complex which affected the complement in identical manner.

If it is the antigen instead of the serum which is reduced to a minimum, say 1 in 160 dilution, the same amount of complement is completely inhibited with dilutions of sera ranging from 1 in 5 to 1 in 40, as shown in Table XXXI.

TABLE XXXI.

Complement Fixation in Ice-chest for $3\frac{3}{4}$ hours, $16\frac{3}{4}$ hours, and 24 hours.

1 vol. Antigen 1 : 160 (Ext. 1 saturated with cholesterin in the cold).
1 vol. Complement ranging from 1 : 50 to 1 : 8.
1 vol. Serum ranging from 1 : 5 to 1 : 160.

Complement Dilution.	Serum Dilutions.						Duration of Ice-chest Fixation.
	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	
1 : 8	c	c	c	c	c	c	$3\frac{3}{4}$ hrs.
1 : 10	c	c	c	c	c	c	
1 : 12	c	c	c	c	c	c	
1 : 15	? c	++++	? c	? c	? c	c	
1 : 25	+	+	+	+	+	+++	
1 : 50	? 0	? 0	? 0	0	? 0	tr	
1 : 8	++++	++++	c	c	c	c	$16\frac{3}{4}$ hrs.
1 : 10	++	++	++	? c	c	c	
1 : 12	+	+	+	++++	c	c	
1 : 15	? tr	? tr	? tr	tr	++++	? c	
1 : 25	0	0	0	0	? tr	tr	
1 : 50	0	0	0	0	0	0	
1 : 8	+++	+++	++++	? c	c	c	24 hrs.
1 : 10	+	+	++	++++	c	e	
1 : 12	tr	tr	tr	+++	? c	c	
1 : 15	? tr	? tr	? tr	+	++++	? c	
1 : 25	0	0	0	0	? 0	tr	
1 : 50	0	0	0	0	0	0	

These experiments, we consider, support our conception both of the role of cholesterin and of the nature of the reacting substances in syphilitic serum. They indicate also that, while the essential reaction, that between the lipoids of organ extract and the syphilitic reacting body, is most probably of a chemical nature, yet its consequences and their effect on the lytic power of complement when present in the reacting mixture are subject to the same laws as govern the reactions between other forms of matter in the colloidal condition.

III.—A Study of the Principles involved in the Wassermann Test. By Arthur Eastwood, M.D.

CONTENTS.

Introduction.

Up to a certain point the problem raised by the Wassermann reaction is relatively simple and may be stated in a few words. Active serum or "complement," when aided by the requisite immune body, dissolves red corpuscles, and may therefore be called a "hæmolysin."* Such hæmolysins may be neutralised by substances, termed "antihæmolysins," which are of various kinds, important examples being (1) specific antigen-antibody combinations, (2) non-specific biological products such as normal serum or egg-albumen, and (3) chemical compounds, such as cholesterin. In the Wassermann reaction it is necessary to arrange and control the test in such a way that an anti-hæmolytic effect is produced when, and only when, syphilitic serum is one of the reagents. The immediate problem is to satisfy this requirement, irrespective of questions as to the precise nature of the "antihæmolysin" which is formed.

If, however, one asks not only for a rule-of-thumb test which appears reliable, but also for a scientific explanation of each step in the technique recommended, the task is extremely difficult. At the present time it is impossible to give a complete explanation; one can do no more than attempt to clear up some of the obscurities, by collecting certain laboratory facts, conjecturing their interpretation, and inviting better explanations.

The Wassermann test is an empirical adaptation of a specific immunity reaction, but differs from such reactions in obvious respects. As to the importance of these differences there is no general agreement. One view is that suitable Wassermann antigens, though not specific in the strict sense, since they may be obtained from normal tissues, behave like specific antigens, and that the reaction conforms to all essential principles which govern true immunity reactions, the extract being analogous to the disintegration products due to the spirochætes. At the opposite extreme there is the view that the test has nothing to do with specific fixation of complement by the union of antigen and antibody, but is merely a non-specific physical or colloidal test, based on the anticomplementary property common to a large variety of substances which cannot possibly be regarded as specific; on this view, the test simply depends on the physical properties of syphilitic serum, which are such that an anti-complementary effect is more readily produced with it than with normal serum. The views of most people, however, are intermediate between these two extremes and compromise, in one way or another, between the specific theory, as defined above, and the non-specific theory; the former is utilised to explain the assumed specificity of "Wassermann substance"

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* I use the term "hæmolysin" in order to emphasise the fact that the result of the test depends upon the hæmolytic activity of complement.

in relation to syphilitic infection, and the latter serves to justify the need for arranging the test as a quantitative differentiation between the anticomplementary capacities of syphilitic, "borderline," and normal sera.

This tendency to compromise, whether it be justifiable or not, assumes many different forms, often taking divergent directions, and consequently makes the literature of the Wassermann reaction particularly difficult to follow.

Much of the literature is concerned with an analysis of the conditions under which, in the test-tube, the hæmolysin termed "complement" is fixed or adsorbed by patient's serum and "antigen." This is very largely a problem of physical and colloidal chemistry and is particularly confusing, because the reagents employed are extremely complex and very little is known about their constitution. To avoid losing one's way, one needs some preliminary ideas as to the nature of "complement," and its relations to antigen and antibody in specific immunity reactions. With such reactions non-specific anticomplementary action may then be compared and contrasted, leaving the way open for the conception of a possible third type of reaction in which the principles of both these two are involved.

There is also an overwhelming amount of literature on "Wassermann substance." Does such a substance exist as a separate entity or is the peculiarity of syphilitic serum due to a physical change only? What is the relationship between the Wassermann reaction and precipitation tests? What part does precipitation play in admittedly specific applications of the complement fixation test? These are only a few of the questions involved. The questions are so many and the issues so complicated that it is very difficult to disentangle any general trend of scientific opinion.

In this study of the literature* I have only attempted to disentangle some of the threads which may, or may not, prove to be useful clues. At present the subject is too obscure for confident interpretation; but, if the obscurity is ever to be cleared up, one must begin by suggesting interpretations at the risk of making mistakes.

I am greatly indebted to Brevet-Col. L. W. Harrison, D.S.O., K.H.P., and to my colleagues, Drs. Griffith and Scott, for their criticisms and encouragement in a difficult task.

As criticism should be based on the principle that the value of an article depends on its protocols and not necessarily on the author's conclusions, one endeavours, in dealing with the literature, first to elucidate the facts and then to interpret them impartially. But immunity research has become deeply and inextricably involved in controversy; even its current vocabulary of technical terms, which is now indispensable, has been coined by the controversialists, and these terms cannot be defined or interpreted without

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* As I assume that the reader is acquainted with the work of such well-known authorities as Harrison, McIntosh and Fildes, and Browning and Mackenzie, I have not quoted from the publications of these investigators.

raising controversial issues. Hence it is impossible to eliminate the controversial equation. However impartially one may desire to criticise, one cannot attempt to interpret laboratory work on immunity, or to discriminate between what is valuable and what is unimportant, without the guidance of some general ideas as to the relative merits of rival theories on the main problems of immunity.

I think the following expresses the leading ideas which have influenced me in preparing this report:—

(1) *Ehrlich's Doctrine.*—In its inception, it has proved a most valuable attempt to explain phenomena of immunity in the light of physiological principles of metabolism. But its elaboration has been based on the idea that the infinite complexity of natural processes must be explained by postulating an inexhaustible supply of different chemical entities, affinities, and side-chains. This idea, whatever merits it may possess, has been pushed too far; it has led to far too free a coinage of chemical conceptions which are merely hypothetical, and the current usage of these terms has caused confusion and impeded progress.

(2) *Bordet's School.*—This school has helped to remove the confusion by showing that immunity experiments *in vitro* can largely be explained by simple laws of physics and colloidal chemistry.

(3) *The Biochemists.*—They have been useful in demonstrating that certain properties of complex media, such as serum, are not, as was formerly thought, due to the existence of special and separable substances contained in the medium but are functions of the medium as a whole.

(4) *Desideratum.*—In interpreting immunity reactions *in vitro* it is desirable to discriminate, as far as possible, between the influence of the essential biological properties of the reagents employed and the influence of the physical and chemical conditions under which the reaction takes place.

Part I.

COMPLEMENT.

Preliminary Considerations.

The literature on complement is very extensive and involved. It may be useful to begin by recalling some general principles of an elementary nature, which are, perhaps, in some danger of being overlooked, owing to the concentration of interest in the endeavour to penetrate more deeply into the problem.

The interaction of active immune serum with antigen may first be considered in relation to the plasma from which it is derived.

In the living body the plasma is "active" in the sense that there is free interchange of its labile molecules, constant building up and breaking down, and constant adjustment and readjustment of equilibrium. This activity is necessary for metabolism and is constantly being renewed, as a necessary consequence of

metabolism.* In other words, assimilation of material introduced into the body involves constant renewal or readjustment of the labile activities of the plasma which result from the chemical and physical interactions of the substances contained in it; there is no one substance which generates the activities of the rest, in the way, for example, in which an engine may activate all the other machinery in a factory.

In the plasma of the immunised animal the process of immunisation is conceivably a special incident of metabolism in which the antigen is merely one out of many extraneous substances introduced into the daily routine of assimilation, one out of many factors which play a part in that process of breaking down and building up labile combinations which constitutes the activity of the plasma. A result of this special incident is the acquirement by the plasma of the special property of "immune body," which assists the active elements of the plasma as a whole to assimilate that foreign intruder, the antigen.

When an animal is bled, the fresh serum is "active" in so far as it retains some of the labile activities of the plasma. But it differs from the circulating plasma in important respects. The activity of the plasma, being part and parcel of the constant changes involved in the metabolism of the living body, is constantly being renewed, whereas the serum, removed from these influences, cannot renew its activity once the latter is destroyed or exhausted. As activity depends on chemical and physical conditions which can only be reproduced in the living body, it may be said that active serum, as such, merely contains the labile remnants of vital processes after the removal of vital influences. These remnants are active, like plasma, in virtue of the chemico-physical condition of their constituents; there is no one constituent in the serum with the special function of generating "activity."

Heating at 56° C. destroys the activity of a serum but does not destroy immune body in the serum from an immunised animal; and it is found that a normal active serum may be artificially converted into an active immune serum by adding to it a small quantity of the heated immune serum. In view of this fact, the heated immune serum which has been added might quite reasonably be termed "complement"; but, perhaps unfortunately, this name has been given not to the immune serum but to the normal active serum.

Following the orthodox use of the term "complement," one must distinguish between complement as a special property

* It is agreed that both plasma and serum contain elements which exercise digestive functions, though questions as to the cellular origin of these elements are still in the controversial stage and in some respects the plasma may be only the vehicle of material elaborated in the tissues. As I do not propose to enter into this controversy, what I say in this report about the assimilative properties of plasma and serum is not intended to imply that I advocate a humoral as opposed to a cellular theory.

and complement as a special substance. It is a recognised fact that complement is a special property of fresh serum, but it is only a hypothesis, and one not accepted by all authorities, that complement is a special substance contained in the fresh serum and behaving, according to one view, as a lipase which modifies cell-membranes. It has never been isolated, and there is not even indirect proof that it exists as a special substance, since heating merely shows that the serum as a whole has been altered, not that a special substance formerly existed but has been destroyed. Hence, when one speaks of complement, its dosage, its inactivation, and so on, it is important to remember that, in strict accuracy, "complement" may be merely a convenient expression for a special property of fresh serum; errors may arise if it is taken for granted that it exists as a special substance, although it must be recognised that this hypothesis cannot be definitely excluded.

A reaction resembling to a limited extent the way in which the living body disposes of a foreign antigen can be reproduced in a test-tube with serum, or a mixture of sera, containing immune body and possessing constituents which are in the chemico-physical condition termed "activity." Here, again, though there is no harm in applying the term "complement" to a normal active serum which has been reinforced with heated immune serum, one must guard against the assumption that complement is a special substance with the function of generating "activity" apart from the other ingredients of the fresh serum. It is also important to remember one of the differences between the test-tube reaction and that which takes place in the living body. In the test-tube the amount of active substances, being limited, is readily used up, *i.e.*, a stable equilibrium is established, and the molecules of the serum cease to be labile; in the living body the active substances are constantly being renewed, and consequently any "fixation of complement" which occurs is of practically no importance under normal conditions. On the other hand, such fixation is a prominent feature of the test-tube reaction, and is used as a demonstration that interaction occurs between antigen and antibody.

It is beyond all question that this use of complement as an indicator, showing whether or no an antigen-antibody reaction has taken place, is of the greatest diagnostic utility, and, apart from other considerations, it may be justified on the biological principle that "complement," as a survival of the plasma, will do its natural and appropriate work if the appropriate material is provided; the disappearance or persistence of the small amount of complement suitable for the test-tube experiment should therefore show whether the material (sensitised antigen) was present or not. Obviously, though accurate quantitative adjustment of the reagents is necessary, the test is qualitative and designed to answer the question: "Is specific antigen (or antibody) present?" by a definite "Yes" or "No"; it would

completely fail in its purpose if the answer were merely a "more or less."

Here one may consider a little more closely in what way complement disappears. Conceivably in the living body antigen, under the sensitising influence of the corresponding antibody, is broken up and assimilated with the aid of the normal constituents of the plasma. At the beginning of the process there is, no doubt, adsorption by antigen of some of the normal constituents of the plasma which have the property of "complement," but the assimilation of antigen cannot be explained by adsorption alone; it must also be of a chemical nature, and the loss of complement which results (here transitory and unimportant) must involve a change due to chemical action (not necessarily proteolysis). In test-tube experiments complement may, of course, be destroyed in a variety of ways, which are unlike anything taking place in the living body; but, in so far as test-tube experiments may be analogous to a vital process of digestion, the loss of complement in the test-tube (here permanent and of conspicuous importance as an indicator) may be expected to be due to similar principles, and to involve not only adsorptive processes but also chemical interaction with and modification of antigen.

In the test-tube, however, particularly when the antigen is not in the form of intact cells, it is by no means a matter of course that the second or chemical part of the digestive process is completed or even initiated. Even under the most favourable conditions, the test-tube experiment is a very imperfect representation of what takes place *in vivo;* and it may very conceivably happen that the only part of the reaction which is demonstrable is the first stage, the adsorption of complement by sensitised antigen. In laboratory terminology, disappearance of complement may be the only evidence that specific antigen and antibody have been brought together.

This last consideration brings into prominence the very important difference, mentioned above, between the processes of immunity *in vivo* and the reactions observed in the test-tube experiments where a diagnosis is made by complement fixation. In the former, the essential problem is to assimilate antigen; complement may usually be left to take care of itself, because the activities termed "complement" are renewable and practically inexhaustible. But in the test-tube the fate of the very limited supply of activity or "complement" becomes all important, and what effect complement has on antigen-antibody appears to be immaterial, provided that this combination has rendered complement incapable of hæmolysing sensitised corpuscles.

In consequence of this artificially exaggerated importance of anticomplementary action, difficulties arise, the chief of which is that the properties of active serum in the test-tube may be very readily destroyed by a large variety of physical, chemical, or biological reagents which have nothing to do with immunity

reactions. Hence the loss of complement means nothing, unless it can be proved that it is not due to any of these extraneous causes. But if it is impossible to demonstrate a specific change in antigen-antibody when destruction of complement is thought to be due to this combination, the exclusion of other possible causes can only be effected by controls showing that no other anticomplementary influences were responsible for the fixation.

When attention is concentrated on this aspect of the problem, viz., the formation of antihæmolysin by means of a particular reagent, one is tempted to assume that the resemblance between the Wassermann reaction and a specific immunity reaction is limited to one feature, the production of an anticomplementary combination. And as anticomplementary combinations may also be formed by a large variety of admittedly non-specific substances, the conclusion may be drawn that the analogy between the Wassermann reaction and specific immunity reactions is useless and, perhaps, illusory. On this view the scientific interests of the problem belong to physics and colloidal chemistry, and it is a question of how far the peculiar properties of syphilitic serum can be explained by the aid of these sciences. But physics and colloidal chemistry cannot be expected to explain everything, because the reagents under investigation are biological products, of highly complex and very imperfectly known constitution, which cannot be reproduced outside the animal body. Therefore the importance of correlating test-tube reactions with biological processes cannot be ignored, and it is particularly necessary to remember that many of the substances split off from biological products by chemical or physical reagents are artefacts which do not represent the constituents out of which these products are built up.

The validity of the Wassermann reaction as a rule-of-thumb test ought, ultimately, to be justified on biological as well as on physico-chemical grounds.

Complement in the Haemolytic System.

The following extracts from work on this subject may help to throw some light on the nature of complement. My personal comments on individual pieces of work are inserted in small type.

Influence of Dilution upon Complement.

In this section I give the work of Kiss and Scheller. It is mainly concerned with the influence of dilution upon the action of complement. Questions as to the individual peculiarities of complements and hæmolytic immune sera are also raised.

Julius Kiss (1909)* investigated a hæmolytic system consisting of washed ox-corpuscles, rabbit *v.* ox amboceptor, and guinea-pig complement. His unit of corpuscles was 1 c.c. of

* *Zeitschr. f. Immunitätsforchung*, Orig. III., p. 558.

a 5 per cent. suspension; the unit of complement was 0·05; and the titre of the immune serum was 0·00125. He used a total volume of 5 c.c. and incubated for two hours at 37° C.

He found that variations in volume did not affect the binding of amboceptor, but that this was not the case with complement. On diminishing the volume, the action of a given amount of complement was increased.

With a unit of corpuscles sensitised with one unit of amboceptor, his results were :—

In a volume of 5 c.c.,	0·05 c.c. complement	gave complete	lysis.
„ „ „ „ „	0·02 c.c. „	„ no	„
„ „ „ „ 2·5 c.c.,	„ „	„ incomplete	„
„ „ „ „ 1·5 c.c.,	„ „	„ complete	„

In another series of experiments, volume (5 c.c.) and complement (0·04) were kept constant, the complement being insufficient to lyse one unit of corpuscles completely, and the amount of corpuscles which could be lysed was determined colorimetrically. The following results were obtained, expressed as units of corpuscles :—

Quantity used :	0·25	0·5	1	2	3	6	12·5	25	50	100
„ lysed :	0·2	0·35	0·8	1·6	2·5	3·5	5	9	9·9	9·3

In the above experiment he purposely used too little complement in order to exclude possible error from excess of complement. In the following experiment the amount of complement was 0·05. The results are expressed as above :—

Quantity used :	0·25	0·5	1	2	4	6	12·5	25	50	100
„ lysed :	0·25	0·5	1	2	4	5·5	10	12	12	15

In another experiment he used single units of corpuscles, amboceptor and complement, and incubated until hæmolysis was complete. He then added 0·25 c.c. of a 100 per cent. suspension of corpuscles, which had been sensitised with a unit of amboceptor for each unit of corpuscles. As this was only a small additional volume, the concentration of complement was not much altered. He found that more than half of the added corpuscles were hæmolysed, *i.e.*, about three units. He obtained the same result when, after the first stage of the experiment, he kept the hæmolysed mixture at 8°—10° C. for 24 hours before adding the second quantity of corpuscles. Incubator temperature, however, hastened the disappearance of complement.

His conclusions were that, given a constant amount of complement, the rapidity and strength of hæmolysis were increased by diminution of volume or, keeping volume constant, by increasing the amount of sensitised corpuscles. The degree of hæmolysis was, within wide limits, independent of the absolute quantity of complement. He considered that these principles were best explained by supposing that complement acted as a catalytic reagent. "Undoubtedly complement is bound, but the principles determining this result are not the same as those which determine the act of hæmolysis."

The above experiments are important as illustrating the influence of dilution, temperature, and time of exposure upon the chemico-physical

activities of fresh serum. But the evidence that these properties are not due to a special chemical compound with quantitatively fixed combining capacities is not conclusive proof that they are due to a special "catalytic reagent."

Scheller (1910)* investigated the action of immune body and complement in a system consisting of sheep's corpuscles, rabbit *v.* sheep antiserum, and fresh guinea-pig serum.

In the first place he found that the action of complement depended on its concentration. When 2 c.c. of a particular suspension of sensitised corpuscles were added to 1 c.c. of a dilution of complement, the minimal amount of complement required to produce complete lysis was $\frac{1}{160}$ c.c.; but, when the bulk of the mixture was increased from 3 to 10 c c. by the addition of 7 c.c. of saline, the minimal amount of complement was increased to $\frac{1}{50}$ c.c. Repeated tests showed that this action of complement followed a general law. When the bulk of the mixture was increased from 3 to 6 c.c., the amounts of corpuscles and amboceptor remaining the same, double the amount of complement was required; this amount was again doubled when the bulk was increased to 12 c.c., and again when the total volume was made 24 c.c. Variations in amount of amboceptor, though affecting the total amount of complement required, did not bring to light any deviation from the law that, with fixed amounts of corpuscles and amboceptor, the action of complement was directly proportionate to its concentration. Scheller has illustrated this statement by tabulating five parallel series of experiments in which the units of amboceptor were 60, 6, 3, 2, and $1\frac{1}{2}$. In each series the law of proportion to concentration (3 c.c.—24 c.c.) was strictly observed. These results were confirmed by experiments with a large variety of hæmolytic amboceptors and complements, the only exception being that less than the theoretical amount of complement was often found to be sufficient when working with high dilutions. In such cases Scheller's explanation was that the relatively large amount of guinea-pig serum required for these high dilutions contained sufficient natural amboceptor to reduce the amount of complement required.

Immune body, on the other hand, was found to be unaffected in its action by dilution. When fixed amounts of corpuscles and immune body were brought together and the liquid obtained after centrifuging was tested for free immune body, it was found that the amount fixed by the mixture was not altered by increasing the volume to twice, four times, or six times that of the original mixture. It was found also that the action of amboceptor was not affected by dilution of the hæmolytic system from 3 c.c. to 12 c.c., provided that the concentration of complement remained the same (1 c.c. amboceptor + 1 c.c. corpuscles + 1 c.c. of 0·1 complement, as compared with 1 c.c. amboceptor + 1 c.c. corpuscles + 1 c.c. of 0·4 complement + 9 c.c. saline).

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* *Centrbl. f. Bakteriol.*, Orig. LVI., p. 120.

Scheller next investigated the action of complement, in given concentration, in relation to the quantity of corpuscles. He obtained results which are illustrated in the following table :—

Quantity of Sensitised Corpuscles. (Volume made up to 9 c.c.)	Hæmolytic Effect of decreasing Amounts of Complement. (Volume made up to 1 c.c.)					
	0·4 c.c.	0·2 c.c.	0·1 c.c.	0·05 c.c.	0·025 c.c.	0·0125 c.c.
½ c.c. -	Complete	Complete	Complete	Nearly complete.	Incomplete.	Trace
1 „ -	„	„	„	„	„	„
2 „ -	„	„	„	„	„	„
4 „ -	„	„	„	„	„	„
8 „ -	„	„	„	„	„	„

With regard to this evidence that the action of complement, in given concentration, was irrespective of the amount of corpuscles to be dissolved, Scheller made the further statement that, in these experiments, the time required for lysis was always the same.

He found, however, that with guinea-pigs' sera containing relatively large amounts of immune body, the tubes with small quantities of sensitised corpuscles showed lysis with smaller concentration of complement than those with large quantities of corpuscles. This he attributed to the influence of the guinea-pigs' amboceptor in the former tubes.

He also observed that the action of complement, as illustrated in the above table, did not hold good if the experiments were arranged with a fixed amount of amboceptor and increasing amounts of corpuscles. Under these conditions the larger amounts of corpuscles required increasing concentration of complement, because they caused relative diminution in the quantity of amboceptor, and on this account more concentrated complement was necessary in order to produce lysis.

The dependance of complement on concentration suggested to Scheller that its action was of a catalytic nature, though it could not be prolonged for an indefinite length of time ; for demonstration of maximum lytic power the total amount of corpuscles to be lysed should be brought into contact with complement simultaneously. Owing to its unstable nature, complement was readily destroyed when present in a medium in which a reaction was taking place ; hence its action, though catalytic, was not continuously renewable.

Scheller also considered that the dependence of the action of complement on concentration, and not on absolute quantity, might serve to explain why, in the process of immunisation, the protection obtained by an increase of immune body did not require, and was not accompanied by, an increase of complement.

Scheller's data are an interesting corroboration of those provided by Kiss, though it appears that other investigators have not been as fortunate as Scheller in obtaining arithmetically precise confirmation of his "law of proportion to concentration."

The idea that the principle of concentration explains why there is no increase of complement during immunisation may be misleading. In the living plasma of the animal undergoing immunisation the labile activities of the plasma may be increased (more breaking down and building up due to the process of assimilating antigen) without alteration in the net balance between catabolism and anabolism, and consequently without evidence of greater activity of the serum of the animal. It is not necessary to postulate that this activity is to be identified with a catalytic substance of unstable nature.

In a second article on the relations of rabbit *v.* sheep antiserum to guinea-pig complement, Scheller, writing in collaboration with Goldschmidt (1911),* recorded the following experiments :—

I.

The units of four immune sera, when tested with four complements (concentration 1 : 30) were found to be :—

	Complement I.	Complement II.	Complement III.	Complement IV.
Immune serum 140 -	1 : 5,000	1 : 5,000	1 : 5,000	1 : 5,000
„ 179 -	1 : 12,000	1 : 10,000	1 : 10,000	1 : 10,000
„ 175 -	1 : 3,000	1 : 1,500	1 : 2,000	1 : 2,000
„ 173 -	1 : 600	(lysis incomplete).	1 : 500	1 : 300

II.

In the following experiment the unit of amboceptor was determined for each hæmolytic serum in the presence of each complement; and it was then ascertained what concentration of a given complement used for the titration of an amboceptor was required to produce lysis when *two* units of that amboceptor were employed. The requisite concentrations were found to be :—

	Complement I.	Complement II.	Complement III.	Complement IV.
Immune serum 140 -	1 : 300	1 : 60	1 : 120	1 : 120
„ 179 -	1 : 30	1 : 30	1 : 60	1 : 30
„ 175 -	1 : 30	1 : 90	1 : 90	1 : 90
„ 173 -	1 : 30	(lysis incomplete).	1 : 90	1 : 90

* *Centrbl. f. Bakteriol*, Orig. LVIII., p. 569.

The authors stated that the above tables were examples of a very large series of experiments, and that the results of all were concordant.

Attention was drawn to the facts that (1) the titre of one and the same immune body might vary considerably when determined with different complements in equal and adequate concentration (1 : 30), and (2) with different immune bodies these variations differed in amount, *e.g.*, one immune body might give the same titre (1 : 5,000) with four different complements, while another gave different titres (1 : 3,000, 1 : 1,500, 1 : 2,000, and 1 : 2,000) with the same four complements. It was therefore concluded that, in addition to the purely quantitative relations between complement and immune body, there were also qualitative differences between individual complements and individual immune bodies, and, owing to these differences, the relations between the two substances were variable. In support of this conclusion it was observed that the rapidity of hæmolysis was not always parallel with the height of the titre, *e.g.*, Complement III., in Table I., always acted more slowly than Complement II. in the same concentration, though Complement III. consistently gave better titres with the variable amboceptors than did Complement II.

Table II. illustrated the variability of the ratio between quantity of amboceptor and concentration of complement required to produce lysis. It showed, for example, that if the unit of amboceptor were determined for a particular optimum concentration of complement, no inference could be drawn as to the amount of complement required for two units of amboceptor, because this depended upon an uncontrollable factor, the idiosyncrasy of the guinea-pig's serum.

It was pointed out that these observations had an important bearing on the Wassermann test. If the amboceptor needed only a small amount of the complement present, considerable fixation would be needed in order to prevent hæmolysis ; but if the demands of amboceptor upon complement were large, slight fixation, possibly within the capacity of a normal serum, would suffice to prevent hæmolysis.

Though the last paragraph illustrates an important aspect of the Wassermann reaction as a quantitative test, corroboration is needed for the general conclusions of Scheller and Goldschmidt as to the existence of important qualitative differences in complements and amboceptors. The observations which they put in evidence are limited to four amboceptors, one of which is too weak to be worth considering, and four complements. Much more extensive protocols are needed to substantiate their views.

Scheller (1911) recapitulated his views in a paper read before a congress at Dresden.* He there stated that his investigations on complement concentration had always been conducted in the water-bath. This he considered to be an

* *Centrbl. f. Bakteriol*, Ref. L., p. 146.

essential point. He had often observed that the minimal dose of complement was smaller when the tubes were at once put into a water bath at 37° C. than when the tubes were first put in the incubator, or left at room temperature, before transfer to the water-bath. In the water-bath the contents of the tubes rapidly attained the temperature of 37° C., but in the incubator this temperature might not be reached even in two hours. Consequently the reaction was much more quickly completed in the water-bath, and complement, which was a labile substance, was more effective; but in the slower process much of the complement was destroyed before the reaction came to an end.

I have inserted the above as it has, perhaps, been overlooked in some of the criticisms which charge Scheller with failing to reach the true end-point in his experiments.

Questions arising out of Scheller's Work.

The articles collected in this section deal mainly with questions arising out of Scheller's work. Attention is called to the importance of considering the rate of reaction. As against the extremist views of Scheller and his opponents, it is urged that physical reactions in general depend on both (*a*) the concentration and (*b*) the absolute quantity of the ingredients, not on (*a*) alone, though (*a*) may be the greater influence with some substances and (*b*) with others. The question of secondary destruction of complement by the products of lysis is discussed.

Liefmann and Andreew (1911)* investigated the action of complement, in constant concentration, as regards the quantity of corpuscles lysed and the speed with which lysis took place.

Using goat *v.* sheep serum and 5 per cent. corpuscles previously sensitised with six units, they found :—

Sensitised Corpuscles.	Saline.	Complement (1 : 10).	Time required for Lysis.	
			In Water-bath.	In Incubator.
0·5	8·5	1	Complete in 20 min.	Complete in 2 hrs.
1	8	1	Complete in 23 min.	Complete in 2 hrs.
2	7	1	Complete in 30 min.	Almost complete in 24 hrs.
4	5	1	Complete in 35 min.	Incomplete in 24 hrs.
8	1	1	Complete in 60 min.	Incomplete in 24 hrs.

* *Zeitschr. f. Immunitätsforschung*, Orig. XI., p. 355.

In another experiment, using the water-bath, as the air incubator took too long to heat large amounts, they estimated the amount of lysis at the end of two hours and found :—

Corpuscles sensitised with six units.	Saline.	Complement (1 : 6).	Lysis.	Quantity of Dissolved Blood, estimated colorimetrically.
0·2 c.c. of 25 per cent.	8·8	·1	Complete in 28 min.	0·2 c.c. of 25 per cent.
0·5 c.c. of 25 per cent.	8·5	1	Almost complete in 2 hrs.	0·48 c.c. of 25 per cent.
1 c.c. of 25 per cent.	8	1	Incomplete in 2 hrs.	0·9 c.c. of 25 per cent.
2 c.c. of 25 per cent.	7	1	,, ,,	1·5 c.c. of 25 per cent.
4 c.c. of 25 per cent.	5	1	,, ,,	2·8 c.c. of 25 per cent.
8 c.c. of 25 per cent.	1	1	,, ,,	5·6 c.c. of 25 per cent.
8 c.c. of 50 per cent.	1	1	,, ,,	5·55 c.c. of 50 per cent.

Reckoned in terms of 5 per cent. corpuscles, the quantities lysed were :—

Quantity used : 1 c.c., 2·5 c.c., 5 c.c., 10 c.c., 20 c.c., 40 c.c., 80 c.c.
,, lysed : 1 c.c., 2·4 c.c., 4·5 c.c., 7·5 c.c., 14 c.c., 28 c.c., 55 c.c.

Continuation of the experiment for 24 hours did not produce complete lysis of the larger quantities.

Taking saponin as a good example of a haemolytic substance which was not a ferment, and comparing it with complement, they obtained the following results :—

I.

Total Volume.	Time required for complete Lysis of 1 c.c. of 5 per cent. Sheep's Corpuscles.	
	With 0·15 c.c. of 1 : 1,000 Saponin.	With 0·5 c.c. Guinea-pig Complement, and 6 units of Amboceptor.
2·5	3½ minutes	6 minutes
5	13 ,,	8 ,,
10	90 ,,	11 ,,
20	Incomplete in 150 minutes	22 ,,

II.

Amount of lysis produced with varying quantities of 50 per cent. sheep corpuscles. Each tube made up to 10 c.c., and kept for three hours at 37° C.

Quantity of Corpuscles.	Amount of Lysis produced with		
	0·4 c.c. of 1 : 1,000 Saponin.	1 c.c. of 1 : 10 Complement ; Corpuscles sensitised with	
		6 units Amboceptor.	2 units Amboceptor.
0·125	Total	Total	Total
0·25	,,	,,	,,
0·5	0·45	,,	,,
1	0·8	,,	0·9—0·95
2	1·1	1·8	1·7—1·8
4	1·8—1·9	3·8	3·5
8	3·6—3·7	7·6	6·8

In comment on the above I may remark that it ought not to be taken for granted that saponin produces hæmolysis in the same way as complement plus immune body, though the experiment may serve the purpose for which the authors intended it, *i.e.*, it shows that the characteristic of complement which is demonstrated above is not enough to prove that it is a ferment.

Ungermann and Kandiba (1912),* in an investigation on the influence of quantitative relations upon the action of antibodies, confirmed, in the main, Scheller's hæmolytic experiments with rabbit *v.* sheep serum and guinea-pig complement. Comparing total volumes of 3 c.c., and 30 c.c., with constant quantities of amboceptor and sheep's corpuscles and graduated amounts of complement, they found that the action of complement was determined by its concentration, and was independent of its absolute quantity. But this rule only held good within certain limits. When the range of variation in total volume was extended by setting up parallel experiments with total volumes of 0·4, 4, and 40 c.c., it was found that with the largest volume less concentration of complement was sufficient to produce lysis than with the smaller volumes. In an experiment recorded, the limits of effective complement action were 0·01 in 0·4 c.c. (1 : 40), 0·1 in 4 c.c. (1 : 40), and 0·1 in 40 c.c. (1 : 400) ; and in another experiment, with total volumes of 1, 10, and 40 c.c., complement was shown to be less effective in the smallest total

* *Arb. a.d. Kaiserlich. Gesundheitsamte*, XL., p. 24.

volume. The details as to the amount of hæmolysis produced, by incubation for two hours at 37° C., were as follows:—

Complement Dilution.	Total Volume.		
	1 c.c.	10 c.c.	40 c.c.
1 : 10	Complete	Complete	Complete
1 : 30	Almost complete	,,	,,
1 : 50	Not complete	,,	,,
1 : 100	Marked	,,	,,
1 : 300	,,	Not complete	Almost complete
1 : 500	Moderate	Marked	Marked
1 : 1,000	Almost nil	Almost nil	Almost nil

The authors stated that they had often observed interference with hæmolysis in small quantities of liquid, and suggested, as two possible explanations to be considered, (1) the quicker clumping and sedimentation of the corpuscles, or (2) the possibility that below a certain absolute quantity of complement even increased concentration would fail to bring about complete hæmolysis.

The general result of their experiments was to confirm Scheller, when they worked with small variations of volume, but to reveal more or less marked divergence from the law that complement acted according to concentration when large differences in volume (1 : 50 or 1 : 100) were compared. They admitted, however, that their results might not be strictly comparable with Scheller's, owing to a difference in technique. Scheller used a water-bath for his experiments, whereas they used an incubator. At all events, their work amply confirmed the fact that the action of complement was profoundly influenced by concentration.

As regards amboceptor, using total volumes (made up with saline) ranging from 2 to 40 c.c., and keeping the concentration of complement constant, they found that, unlike complement, it acted, within wide limits, according to absolute quantity and was not affected by variations in concentration.

In physical chemistry, the authors remarked, one could not say that certain substances acted exclusively according to concentration and others exclusively according to absolute quantity, but it was possible to say that, within certain limits, the action of some substances was predominantly determined by either the one principle or the other. If chloroform were shaken up with ether and water, the chloroform was taken up by the ether almost quantitatively and was little influenced by the proportion of water present (*cf.*, interaction of amboceptor and antigen). But, in a mixture of alcohol, ether, and water, the amount of alcohol taken up by the ether was very largely determined by the concentration of the alcohol (*cf.*, interaction of complement and corpuscles).

The work by Ungermann and Kandiba serves as a corrective to the extremist attitude of Scheller.

In the experiment where they found 0·1 c.c. of complement in 40 c.c. as effective as 0·1 in 4 c.c., there can be no question of more natural amboceptor being present in the higher dilution. The use of an incubator instead of a water-bath would involve a lower rate of the intermolecular activity constituting complementary action, *i.e.*, less rapid deterioration when the total volume was large. On the other hand, when the total volume was very small, complement would be brought up to 37° C. more quickly, and hence would deteriorate more rapidly; moreover, disintegration products of the reaction would be more concentrated and, therefore, more effective in combining with the active serum.

The analogy between the effect of ether (= corpuscles) on (1) chloroform (= amboceptor) and (2) alcohol (= complement) is suggestive, but does not fully express the difference between amboceptor, which is thermostable, and therefore does not act like a labile substance, and complement, the activity of which is due to its labile elements.

Schlemmer (1916),* in an investigation on the action of amboceptor and of complement, called attention to the observations of Kiss, which have been quoted on p. 87. A dose of complement was taken, which, in given concentration, just failed to produce complete lysis of a small quantity of sensitised corpuscles; then in a parallel experiment, with the same concentration of complement, a very much larger quantity of similarly sensitised corpuscles was used. Comparing the two experiments, Kiss found that, though hæmolysis was incomplete in each, the amount of lysis was very much greater in the second than in the first.

Both with amboceptor and with complement Schlemmer observed that dilution affected the rate of the reaction. In the case of hæmolytic amboceptor, which, it was admitted, acted according to absolute quantity, he showed that, with total volumes of 1·5 and 15 c.c., the amount of amboceptor which was bound in 10 minutes was greater in the smaller volume.

He thought it particularly important to consider rate of reaction in relation to complement, because hæmolysis was accompanied by an anticomplementary reaction which, in his view, commenced as soon as the first corpuscles had been lysed and was not "secondary" in the sense of being delayed until the hæmolytic process was completed. Thus complement was used up in two portions, the one being employed in the lysis of antigen and the other being destroyed in the anti-complementary or "methæmolytic" part of the reaction. Hence the quicker the combination between complement and sensitised cells, the greater was the portion used for hæmolysis and the less was there left for the "methæmolytic" reaction. Therefore, in order to hæmolyse the largest possible amount of blood with the smallest possible amount of complement, the rate of reaction must be as rapid as possible. This rate would be influenced by the amount of antigen (corpuscles) in given volume, rapidity being favoured by reduction of the space

* *Arb. a. d. Kaiserlich. Gesundheitsamte*, L., p. 431.

separating complement and sensitised cells; *i.e.*, the reaction should take place in the smallest possible volume. This, he considered, was the principle underlying Scheller's experiments; it held good only within certain limits; if the volume was increased above these limits, a further increase in the amount of complement was no longer necessary. A limited quantity of corpuscles could not use up more than a limited amount of complement in the "methæmolytic" reaction; if this limit was reached, further increase in volume of fluid ceased to have an inhibitory effect on the reaction.

The second method of reducing the space separating complement and amboceptor was, whilst keeping complement in the same concentration, to present to it a larger number of amboceptors anchored to cells. This was possible in two ways:—(1) *By stronger sensitisation, i.e., by attaching as many amboceptors as possible to the same cell.* This corresponded to the well-known fact that strongly sensitised corpuscles needed less complement for lysis than weakly sensitised. If, however, the number of amboceptors anchored to a corpuscle exceeded the optimum, individual corpuscles, owing to their richness in amboceptor, attracted to themselves more complement than was necessary for their lysis, so that no more complement was left for other corpuscles. This was in conformity with the observation that with too strong sensitisation the amount of complement must again be increased to produce complete lysis. (2) *By increasing the number of corpuscles in a given volume without increasing the degree of sensitisation.* The consequence would be that complement, being able to unite with and hæmolyse more corpuscles in a given time, would be used up in geater quantity for the purpose of hæmolysis, whereas with more protracted union and lysis a larger amount of complement would be left for the methæmolytic reaction.

Schlemmer's conclusion was that Ehrlich's conception of the chemical union of complement and amboceptor was correct, apparent discrepancies being explained by the concurrent "methæmolytic" reaction.

The "methæmolytic" hypothesis seems intended to support Ehrlich's general principles of immunity as opposed to Bordet's. But it is not necessary in this connection to become involved in that controversy, and certainly the importance of fixation of complement by adsorption cannot be ignored.

The simpler explanation, also suggested in my comment on Liefmann and Cohn (p. 104), may suffice, viz., union of complement with disintegration products of the reaction. The rate of reaction is undoubtedly important, but in this connection also the simpler explanation may be sufficient, taken in conjunction with the circumstances to which Schlemmer calls attention as favouring rapidity of reaction.

Leschly (1916)* in the first of a series of articles on the nature of complement, criticised Scheller on the ground that

* *Zeitschr. f. Immunitätsforschung*, Orig. XXIV., p. 499.

the greatest quantity of blood corpuscles which could be *completely* hæmolysed by a given unit of complement was very little in excess of the amount used to determine the titre. With ascending amounts of corpuscles and a fixed amount of complement, it was usually found that the quantities hæmolysed, though absolutely greater, were relatively smaller.

In the following experiment the titre of guinea-pig complement for 0·1 c.c. of 5 per cent. corpuscles was 0·01. The titre of amboceptor was 0·0008. The corpuscles, in 25 per cent. suspension, were sensitised for one hour at 37° C. and washed. The total volume in each tube was 2·5 c.c., and the amount of complement used in each was 0·01 c.c. The results were as follows, the corpuscles being calculated in each case as a 5 per cent. suspension for convenience of comparison :—

Amount of Corpuscles used.	Amount lysed.	Percentage lysed.
0·1 c.c.	0·1 c c.	100
0·2 c.c.	0·19 c.c.	95
0·5 c.c.	0·44 c.c.	88
1 c.c.	0·8 c.c.	80
2 c.c.	1·05 c.c.	52·5
5 c.c.	1·33 c.c.	26·6
10 c.c.	1·8 c.c.	18

Leschly stated that this was an example of the usual result with a fixed amount of complement, but sometimes, when small amounts of complement were used, the reduction in the amount of lysis was absolute as well as relative. He found that the relative amounts lysed, when large quantities of corpuscles were employed, varied considerably in different experiments.

He also endeavoured to show in another way, by determining the titre of amboceptor (in the same volumes) for different quantities of blood, that the amount of complement bore an important relationship to the amount of corpuscles.

The total volume in each case was 5 c.c. The corpuscles were made up to 2 c.c. In a control test it was found that 0·2 c.c. of the guinea-pig serum alone produced no hæmolysis. Results :—

5 per cent. Corpuscles.	Titre of Amboceptor with		
	0·05 Complement.	0·1 Complement.	0·2 Complement.
2 c.c.	>0·1	>0·1	0·004
1 c.c.	>0·1	0·0016	0·0016
0·5 c.c.	0·0008	0·0008	0·0008
0·25 c.c.	0·0004	0·0004	0·0004
0·125 c.c.	0·0002	0·0002	0·0002

In another experiment he determined the titre of 20 complements in four different volumes; 0·5 c.c. of 5 per cent. corpuscles sensitised with two units of amboceptor was used. Readings were taken after two hours at 37° C. and 20 hours at 0° C. I select five of his results as a sample of the whole.

No. of Complement.	Complement Titre in volume of			
	1·25 c.c.	2·5 c.c.	5 c.c.	10 c.c.
1	0·035	0·035	0·04	0·05
5	0·03	0·025	0·03	0·05
10	0·025	0·02	0·04	0·08
15	0·013	0·013	0·02	0·03
20	0·016	0·016	0·025	0·05

He noted that the influence of dilution was not the same with different complements.

Leschly claimed that the above results were notably different from Scheller's, and that the divergence from Scheller was still more marked if more than two units of amboceptor were taken. In the following experiment he used an amboceptor of titre 0·001 and 0·5 c.c. of 5 per cent. corpuscles.

Units of Amboceptor.	Complement Titre in volume of			
	1·25 c.c.	2·5 c.c.	5 c.c.	10 c.c.
1	0·025	0·04	0·05	0·08
2	0·016	0·016	0·025	0·05
5	0·016	0·016	0·016	0·03
10	0·016	0·016	0·016	0·025

As regards the relations of complement to amboceptor, Leschly maintained that, provided both complement and corpuscles were fresh, the amount of hæmolysis attained with a certain amount of complement, or of amboceptor, was only slightly dependent on the amount of the other factor, if these amounts exceeded what was requisite for total hæmolysis. He conceded, however, that hæmolysis with one unit of amboceptor might sometimes be slightly less than with $1\frac{1}{2}$—20 units, and that some immune sera might exhibit an inhibitory effect when as many as 20 units were used.

He dissented from the statements of Scheller and Goldschmidt as to the qualitative differences of individual amboceptors and complements, and thought it questionable whether the titres found by these investigators were really the true titres. Using 20 amboceptors and more than 50 guinea-pig

sera, he failed to find any significant variations in titre which would point to such qualitative differences.

He recognised, however, that there was a difference in the rapidity with which different amboceptors acted, and also that the action of complement was made slower by dilution. But this relationship between concentration and rapidity of action did not, in his opinion, justify the statement that, in its end result, the action of complement was determined by concentration and not by absolute volume.

Leschly's article is mainly controversial. One may concede that Scheller is inclined to over-emphasis; but the importance of concentration cannot be denied or explained away.

Kaup (1917) discussed the hæmolytic system in a critical review of the Wassermann reaction which he wrote in collaboration with Balser, Hatziwassiliu, and Kretschmer.*

As regards amboceptor, he agreed with previous investigations which had shown that (1) increase of corpuscles, other conditions being constant, required proportionate increase of amboceptor, and (2) amboceptor acted according to absolute quantity and not according to concentration. He thought the relationship between quantity of amboceptor and quantity of complement obeyed a regular law. With a fixed amount of corpuscles, the amount of complement required for complete lysis was essentially greater with one unit of amboceptor than with 2 or 3 units of the latter; but greater excess of amboceptor (from 4 to 20 units) did not progressively reduce, to any marked degree, the amount of complement required. Rapidity of lysis increased with ascending doses of amboceptor, within limits; when doses of 4 to 6 units were exceeded, the differences were not progressive, to any significant extent. Thus, with from 4 to 6 doses of amboceptor the minimal limit of complement was reached, and the same held good, *vice versâ*, with excess of complement and minimum of amboceptor. In the relationship between complement and amboceptor there were quantitative differences between individual sera, but all conformed to the above general principle; nor was the relationship disturbed to any important extent by the possible presence of normal amboceptor in the guinea-pig or other serum.

Kaup agreed with Scheller that the quantity of sensitised corpuscles which could be hæmolysed with a fixed quantity of complement varied within very wide limits. In his experiments on this point, Kaup used varying quantities of corpuscles (each unit being sensitised with 4 units of amboceptor), and decreasing doses of complement; the total volume was made up to 2·5 c.c. The tubes were incubated for half an hour and then read; a second reading was taken after they had remained at room temperature for one hour, and it was then found that lysis had

* *Archiv f. Hygiene*, LXXXVII, pp. 1–175.

proceeded somewhat further. The following is from his Experiment II. (Second reading):—

Complement.	Percentage of Corpuscles.			
	2·5.	5.	7·5.	10.
0·020	Complete lysis	Complete lysis	Complete lysis	Complete lysis
0·012	,, ,,	,, ,,	,, ,,	,, ,,
0·010	,, ,,	,, ,,	,, ,,	Almost complete inhibition.
0·008	,, ,,	Very slight inhibition.	Slight inhibition	Complete inhibition.
0·006	Slight inhibition*	Definite inhibition.	Complete inhibition.	,, ,,
0·004	Complete inhibition.	Complete inhibition.	,, ,,	,, ,,
0·002	,, ,,	,, ,,	,, ,,	,, ,,

* In this table "inhibition" (Hemmung) is not quite accurate. "No lysis," "incomplete lysis," &c. would be better.

As opposed to Scheller, Kaup maintained that complement acted according to absolute quantity, and not according to concentration. Kaup compared the titre of complement in volumes of 1·25, 2·5, 5, and 10 c.c., excess of amboceptor and quantity of corpuscles being constant. He observed that incubation for half an hour was not long enough for the completion of the reaction, and also that the differences in the titre of complement in different volumes became less when the excess of amboceptor was increased. Examples:—

(1) Titre of 3 complements. Incubation for half an hour, followed by 1½ hours at room temperature before reading.

(*a*) 2 units of amboceptor.

Complement.	Volume.			
	1·25.	2·5.	5.	10.
I.	0·012	0·014	0·025	0·035
II.	0·012	0·014	0·02	0·04
III.	0·014	0·014	0·02	0·04

(*b*) Repetition of above with 4 units of amboceptor.

I.	0·01	0·01	0·016	0·025
II.	0·01	0·01	0·014	0·025
III.	0·01	0·01	0·016	0·025

(2) Titre of 2 complements. Incubation for 2 hours, followed by 16 hours at room temperature.

(*a*) 2 units of amboceptor.

Complement.	Volume.			
	2·5.	5.	7·5.	10.
I.	0·02	0·025	0·035	0·035
II.	0·03	0·035	0·045	0·04

(*b*) Repetition of above with 8 units of amboceptor.

I.	0·008	0·008	0·008	0·008
II.	0·008	0·008	0·01	0·01

Kaup's general conclusion was that the action of complement was not of a ferment-like or catalytic nature.

I have quoted Kaup's article as it is one of the most recent general reviews of Wassermann technique and has some bearing on questions of concentration in the hæmolytic system.

Mechanism of Complement Fixation.

This section discusses the relation of hæmolytic immune body to complement, and raises the question as to the desirability of distinguishing between the influence of adsorption and the influence of chemical action in causing the "fixation" of complement.

Liefmann and Cohn (1911)* referred to experiments of Sachs and Bolkowska with a serum (rabbit *v.* ox corpuscles) of relatively low titre (titre 0·0015 with 0·1 complement and 1 c.c. 5 per cent. corpuscles, in total volume of 5 c.c.). With increasing doses of amboceptor, it was found that, at 0° C., when 20 doses or more were used, the corpuscles fixed practically all the mid-piece of complement, leaving the end-piece free in the fluid; and similar, but less complete, results were obtained with smaller amounts of amboceptor. These results were apparently inconsistent with previous observations of Liefmann and Cohn, who, using a high titre serum (goat *v.* sheep corpuscles), had found that, even at 37° C. and with large quantities of amboceptor, only a small amount of mid-piece was fixed. When, however, they repeated Sachs' experiment with a serum (rabbit

* *Zeitschr. f. Immunitätsforschung,* Orig. XI., p. 166.

v. sheep) of a titre (0·00125) about the same as that used by Sachs, they got the same results as those obtained by Sachs and Bolkowska. On returning to their strong serum and making a parallel experiment, but with 75 units instead of 20 units, which was the dose used of the weaker serum, they found that mid-piece was far from being completely taken up. This last result was confirmed on repeating the experiment and washing the corpuscles after sensitising. More complement was bound, however, when they used a goat serum of lower titre. The explanation which they suggested was that the more potent serum, being used in less amount, contained less non-specific anticomplementary substance ("Bordet's antibodies"). At all events, they thought their experiments justified the conclusion that the binding of mid-piece, in quantitative proportions, was not a necessary preliminary to hæmolysis.

The current assumption that complement was bound to the amboceptor-laden cells was, they considered, difficult to reconcile with the facts that (1) blood cells charged with moderate amounts of amboceptor did not bind any demonstrable amount of mid-piece, and (2) whilst hæmolysis was accompanied by disappearance of complement, the disappearance of much the greater part of it was subsequent to hæmolysis. For example, they took a hæmolytic system in which 3 c.c. of 5 per cent. sheep corpuscles, with $\frac{1}{400}$ goat amboceptor (15 units), was just sufficient to use up 1 c.c. of 1 : 10 complement when incubated for $1\frac{1}{4}$ hours. If, after that period, 1 c.c. of sensitised corpuscles was added, they were not lysed, or were not lysed completely. But if 1 c.c. of corpuscles was added immediately after the lysis of the first 3 c.c., which took five minutes, it was lysed completely in six minutes. Again, if immediately after the lysis of the first 3 c.c., the tubes were kept for $1\frac{1}{4}$ hours at 0° C. or at 20° C., and then 1 c.c. of sensitised corpuscles was added, complete lysis of these took place in three minutes. The authors concluded that a temperature of 37° C. was necessary to cause the disappearance of complement, and that this disappearance was subsequent to, and perhaps in consequence of, its hæmolytic action. In this latter respect they compared the behaviour of rennet. Their theory was that, as a secondary result of its action, complement, with the aid of amboceptor, formed an anticomplementary substance out of antigen. This, they thought, explained why (1) with strongly sensitised corpuscles a small amount of complement was sufficient to produce hæmolysis but a good deal of this complement was destroyed, whereas (2) with weakly sensitised corpusles the reverse was the case.

Whilst the amount of complement which disappeared depended to a large degree on the amount of amboceptor used, it depended to a still greater extent on the amount of corpuscles. For example, in an experiment with 5 per cent. sheep corpuscles (sensitised with 15 units of amboceptor and

then washed) and 1 c.c. of 1 : 10 complement in each tube, they found :—

Corpuscles.	Saline.	Lysis complete in	Subsequent Incubation for	Corpuscles added.	Lysis produced.
1 c.c.	3 c.c.	5 mins.	1¼ hrs. at 37° C.	1 c.c.	Complete in 7 mins.
2 c.c.	2 c.c.	5 mins.	„ „	1 c.c.	Partial in 1 hr.
3 c.c.	1 c.c.	5 mins.	„ „	1 c.c.	No lysis in 2 hrs.
3 c.c.	1 c.c.	5 mins.	(No pause)	1 c.c.	Complete in 6 mins.
4 c.c.	—	7 mins.	——	—	——

Their conclusion that complement is not bound in quantitative proportion is supported by general evidence that different quantities of the antigen-antibody complex may require the same minimum amount of complement for their complete digestion.

In place of the postulate of a secondary or methæmolytic reaction, with creation of anticomplementary substance out of antigen, it might suffice to assume that complement goes on acting, and that there is union of the labile elements of complement with disintegration products of the reaction.

The activity of fresh serum may be abolished in two ways: (1) by adsorption, involving removal of "mid-piece," or (2) by chemical action, involving loss of "end-piece." Perhaps the differences found by Liefmann and Cohn between the effects of strong and weak immune sera may be due to the formation of a better adsorption compound when the cells were sensitised with larger amounts of sera. If "mid-piece" and "end-piece" be regarded not as actual substances but as expressing functions of active serum, the former may be said to be concerned with adsorption and the latter with chemical action.

Bordet (1912)* denied that complement acted catalytically, and maintained that it must be absolutely fixed in order to produce lysis. There was no such phenomenon as a secondary "methæmolytic reaction," but the same process, fixation of complement by the stroma, continued after enough complement for the production of lysis had been fixed. Corpuscles were capable of absorbing† widely different amounts of amboceptor, and, when they were strongly sensitised, relatively little alexin (complement) was required for their lysis. Again, a quantity of amboceptor sufficient for the lysis of a given quantity of corpuscles would only hæmolyse about half that amount if the corpuscles were added fractionally, because the first portions of corpuscles would take up much more amboceptor than they needed for lysis, and none would be left for the last portions. (*Cf.* fractional absorption of a dye with blotting paper.)

He called attention to the fact that the more highly corpuscles were sensitised the more marked was their affinity for alexin and the greater the energy with which they absorbed it. Hence,

* *Zeitschr. f. Immunitätsforschung*, Orig. XII., p. 601.

† Personally, I prefer "adsorption" to "absorption," but when authors use the latter word I have not thought proper to alter it when quoting from their work.

highly sensitised corpuscles overcame much more easily antagonistic influences, which, as they tended to maintain alexin disseminated in the liquid, were adverse to fixation.* Similarly, too great dilution of alexin, as Scheller observed, was unfavourable to absorption, the factors which determined hæmolysis being not merely the absolute quantity of alexin but also its concentration. Between the claims of the sensitised corpuscles and those of the surrounding liquid for the possession of alexin there was established a state of equilibrium, and the consequent apportionment of alexin depended on the degree of sensitisation of the corpuscles. When feebly sensitised they were only able to overcome antagonistic influences sufficiently to absorb a relatively very small portion of the alexin present. Thus, as he had shown with Gay, after a liquid containing alexin had hæmolysed the maximum of weakly sensitised corpuscles (as shown by the absence of further hæmolysis on the addition of similar corpuscles), it was erroneous to suppose that it had become devoid of alexin, because it was still capable of hæmolysing on the addition of more strongly sensitised corpuscles.†

Bordet also referred to an experiment made by Liefmann and Cohn, who compared the effect of adding fractionally to a given quantity of alexin, until no more were hæmolysed, (*a*) weakly sensitised cells and (*b*) strongly sensitised cells; the total volume hæmolysed was greater in (*a*). This apparently paradoxical result Bordet explained as due to the differences, mentioned above, between weakly and strongly sensitised corpuscles in their affinity for alexin.

Whilst agreeing with Bordet that it is not helpful to regard complement as a catalytic reagent, one need not feel compelled to adopt the alternative view that complement is a reagent which must be "absolutely fixed." If complement is not a special substance but an expression of the labile activities of serum or "a chemico-physical function of the elements of the serum" (Hecht), "fixation" will mean loss of this activity or function, but will not necessarily mean loss of a particular substance. The facts adduced by Bordet seem to be covered by the statement that digestion of antigen-antibody with the aid of active serum takes place more readily when (1) antigen is highly sensitised by antibody, and when (2) the active serum is present in a concentration above the minimum requisite; but the activity of the serum is impaired (*a*) by increasing the amount of immune body, and thereby increasing the adsorptive capacity of sensitised cells for alexin before lysis, and (*b*) by expending itself more readily in further interaction with disintegrated cells after lysis. Bordet's conception of sensitised cells and the surrounding liquid as "antagonistic forces" competing for the possession of alexin seems to be based on the theory that the mechanism of the reaction is purely one of adsorption.

Idiosyncrasies of Complement.

I now quote from a few of the articles illustrating the importance of considering idiosyncrasies of complement in relation to the Wassermann reaction.

* This tendency of alexin to maintain itself in the liquid, Bordet remarked, was greatly facilitated by such "antihæmolytic" or "antibactericidal" substances as sodium citrate and heated serum.

† Bordet employed this fact as an argument against the multiplicity of alexins in the same serum.

Noguchi and Bronfenbrenner (1911)‡ made observations on the variations in the complement activity and fixability of the serum of 41 guinea-pigs. The amount of complement fixed was determined by the colorimetric method of Madsen. The serum was a mixture from several untreated cases of secondary syphilis and was inactivated before use. Their antigen was the acetone-insoluble fraction of lipoids from a human liver.

Complementary Activity.—With sera which had been in contact with the clot for 20 hours at 0° C. the strongest and weakest were in the ratio of 0·015 to 0·04; with the same sera after 46 hours at 0° C. the strongest was 0·013 and the weakest 0·06. A large number gained in complementary activity by standing for the longer period, but some became weaker.

Fixability.—The amount of serum fixed by given constant quantities of syphilitic serum and antigen varied much more markedly than were the variations in complementary activity. One serum failed altogether to be fixed. One was so easily fixed that 0·24 c.c. disappeared (corresponding to 9·6 complement units), whilst the average quantity fixed was only 0·098 c.c. (corresponding to 4·64 complement units). "There is no "definite relationship between the complementary activity "and the fixability of a given specimen of guinea-pig serum."

The authors considered that the above facts showed the necessity for the utmost precaution in conducting the Wassermann reaction. "No quantitative work is possible with the "complement fixation reaction unless the experimenter is "capable of determining the fixability of the serum in use." Hence, long ago, Noguchi had advised employment of a mixture of sera from more than two guinea-pigs. The authors analysed their results as follows :—

Number of units of complement fixed.	Number of specimens.	
0	1*	Zone of non-fixability (2·4 per cent.).
1·5	1	
2	0	
2·5	2	Zone of inferior fixability (19·5 per cent.).
3	1	
3·5	4	
4	6	
4·5	9	Zone of normal fixability (48·7 per cent.).
5	5	
5·5	3	
6	5	
6·5	1	
7	1	
7·5	1	Zone of super-fixability (29·2 per cent.).
8	0	
8·5	0	
9	0	
9·5	1†	

* Not abnormal in complementary activity.

† Usual titre of activity, but unusually rapid deterioration in 46 hours. (Another serum showing rapid deterioration was not abnormal in its fixability.)

‡ *Journ Exper. Med.*, XIII., p. 69.

Kotzewaloff (1913)* stated that, up to the year 1911, it was the custom at the Bacteriological Institute at Charkow to use a fixed quantity of complement (0·05 c.c. in a total volume of 2·5 c.c.) without titration, and to titrate amboceptor, three units of the latter being used in the final test. Sometimes, however, discrepant results were obtained, and these, he was led to believe, were due to variations in complement.

The use of excess of amboceptor was not, in his experience, a safeguard against the occurrence of such irregularities in the results of the test. For example, on titrating a complement with increasing amounts of amboceptor, viz., 1 : 3000 (titre), 1 : 2000, 1 : 1000 and 1 : 200, he found that, at the end of an hour's incubation, the unit of complement required to produce complete lysis was in every case the same (0·02 c.c.); the only difference exhibited by the larger quantities of amboceptor was, as shown by the half-hour readings, that they acted more rapidly. As several repetitions of this experiment led to similar results, with only slight variations, the author attached no importance to the quantitative influence of amboceptor as a corrective of complement, but always used three units of the former.

He found that complements often differed markedly in their deviability by antigen. As an example, he quoted an experiment with two complements, A and B, and two antigens. To antigen (0·06, 0·04, 0·02 and 0·01 c.c., each made up to 0·5 c.c.) he added 0·1 c.c. of normal serum and 0·05 c.c. of complement (each made up to 0·5 c.c.) and 1 c.c. of $2\frac{1}{2}$ per cent. sensitised corpuscles. Both complements gave complete lysis with 0·01 c.c. of antigen; complement B also gave complete lysis with each of the higher concentrations of antigen, but complement A gave complete inhibition with 0·02, 0·04, and 0·06 of the one antigen, and, with the other antigen, results ranging from complete inhibition with 0·06 to partial inhibition with 0·04 and almost complete lysis with 0·02. On another occasion he found that a complement (titre without antigen, 0·02) gave complete inhibition in the usual dose (0·05) when titrated with four antigens; another complement, titrated with the same antigens, gave a titre of 0·03. Lytic capacity, therefore, was not parallel with deviability.

In a further investigation of the variability of complement, he used 10 guinea-pigs, each of about equal weight (350–400 grammes) and kept under the same conditions. About 1 c.c. of serum was obtained from each at intervals of 1–2 weeks during a course of 3 months, throughout which period the animals increased in weight. Following the routine adopted in his Institute, the complements were tested as follows :—

Titration of Complement per se.

0·5, 0·4, 0·3, 0·2, and 0·1 of complement (1 : 10) were each made up to 1·5 c.c., added to 1 c.c. of $2\frac{1}{2}$ per cent. sensitised corpuscles, and incubated for $\frac{1}{2}$ hour.

* *Centrbl. f. Bakteriol*, Orig. LXX.

Titration of Complement with Antigen.

The same amounts of complement, each made up to 1 c.c., were added to the titre dose of antigen (with this antigen 0·02 c.c.), which was made up to 0·5 c.c., and the mixtures were incubated for 50 minutes; then 1 c.c. of $2\frac{1}{2}$ per cent. corpuscles sensitised with 1 : 600 amboceptor was added; incubation for $\frac{1}{2}$ hour followed by room temperature for 5–6 hours.

Results of Complement Titration.

{ Upper figure = titration of complement alone.
{ Lower ,, = ,, ,, with antigen.
0 = no hæmolysis with 0·1 of complement.

Number of Guinea Pig.	Date of Test.								
	26 Jan.	9 Feb.	16 Feb.	23 Feb.	10 Mar.	22 Mar.	5 Apl.	12 Apl.	19 Apl.
1	0·01	0·01	0·02	0·02	0·02	0·02	0·02	0·03	0·02
	0·03	0·03	0·03	0·03	0·03	0·03	0·02	0·06	0·02
2	0·01	0·01	0·01	0	0·01	0·015	0·01	0·02	0·02
	0·02	0·02	0·03	0	0·03	0·02	0·02	0·04	0·03
3	0·01	0·02	0·02	0·1	0	0	0·05	0	0
	0·02	0·03	0·02	0·2	0	0	0·1	0	0
4	0	0·04	0·03	0·02	0·02	0·02	0·02	0·03	0·02
	0	0	0	0·02	0·03	0·02	0·02	0·03	0·02
5	0·01	0·01	0·02	0·02	0·01	0·02	0·02	0·03	0·02
	0·03	0·04	0	0	0·03	0·02	0·02	0·05	0·02
6	0·01	0·01	0·02	0·02	0·02	0·02	0·02	0·02	0·02
	0·03	0·02	0·03	0·02	0·03	0·03	0·03	0·05	0·02
7	0·01	0·01	0·02	0·01	0·01	0·03	0·02	0·03	0·03
	0·03	0·02	0·02	0·02	0·02	0·04	0·03	0·06	0·03
8	0·01	0·015	0·01	0·01	0·01	0·03	0·03	0·02	0·02
	0·02	0·02	0·03	0·02	0·02	0·03	0·03	0·03	0·03
9	0·01	0·01	0·03	0·02	0·02	0·02	0·04	0·03	0·02
	0·02	0·02	0·03	0·02	0·02	0·02	0·06	0·05	0·02
10	0·01	0·01	0·02	0·015	0·02	0·02	0·02	0·02	0·02
	0·02	0	0	0	0	0	0	0	0

Subsequently, Kotzewaloff did some experiments in which he titrated complement in the presence of both antigen and normal serum. He found that the addition of normal serum usually furthered hæmolysis. In the experiments which he has quoted, it reduced the titre of complement from 0 03 to 0·02. But, exceptionally, he found, the addition of normal serum had the reverse effect. His conclusion was that, though it was essential to titrate complement, and to perform this titration in the presence of antigen, the addition of normal serum to this control test was more confusing than helpful.

Comments on the Properties of Complement.

In attempting to co-ordinate laboratory observations on the hæmolytic system, it is useful to distinguish between two aspects of the reaction; (1) in some respects it may be conceived to be an imitation of a natural process of digestion *in vivo*; (2) to a large extent, the test is made under conditions which are artificial, in the sense that they are imposed by laboratory requirements and have no counterpart in natural processes.

When alien corpuscles are introduced into an animal, digestion takes place, aided by the immune body which is elaborated. The natural process of digestion does not stop at hæmolysis, but goes on to further decomposition of the corpuscles after they have been lysed. In the test-tube there is a tendency in the same direction. The active serum, in association with the appropriate amount of immune body, continues, after lysis has been produced, to act on the stroma of the cells until its energies have been exhausted. And, as the amount of active serum is limited, when more energy is expended in attempting to digest laked corpuscles, there is less available for hæmolysing intact corpuscles. This is one of the factors of importance in experimental estimates of the quantity of corpuscles which can be laked by the same amount of active serum under varying conditions. Such experiments show that complement is not simply a hæmolysin and nothing more, and that it would be seriously misleading to assume that its lytic property is its only function of importance in the test-tube which is put up to show the presence or absence of lysis. When this fact is recognised, it is not necessary to add to the confusion by postulating that, in the course of a hæmolytic experiment, part of the hæmolysin is destroyed by a newly formed "antihæmolysin."

Digestive processes differ in rapidity. They may differ not only in different animals of the same species, but also in the same animal under different conditions of metabolism. It is natural to find similar differences in the active properties of fresh guinea-pig sera. They differ in rapidity of action and, when they are similar in other respects, which is not always the case, the more rapidly acting are the more strongly hæmolytic. It is useful to remember, therefore, that natural differences in rate of reaction form an important part of the reasons why complements differ in titre.

Similar differences in rapidity of action may easily be produced artificially in the same serum by varying the dilution; the same quantity of serum will act more slowly when it is diluted than when it is concentrated. If this simple fact were given more consideration, it might go far to explain the experimental difficulties which have given rise to elaborate controversies as to whether complement acts quantitatively or

according to concentration. And, as it is not necessary to assume that complement is a special substance, it may not be requisite to raise what is perhaps an artificial difficulty by debating whether this hypothetical substance is "absolutely fixed" or behaves like "an unstable catalytic ferment." Conceivably some active sera proceed, after adsorption by the sensitised antigen, to the second stage of their activity—the exercise of their digestive function—more rapidly than others. These would then be found to have been exhausted on the addition of the hæmolytic indicator.

Digestive processes may also differ qualitatively. The labile elements which take part in the process are not necessarily identical either in the same species or in the same animal under different conditions. Similar differences are found in the labile elements of active sera, and the consequence is that the chemico-physical condition termed "activity" is terminated more readily with some sera than with others. The laboratory expression for this fact is that some complements are "inactivated," by contact with antigen and antibody, more readily than others.

There are thus two variable factors in active serum, the rate of reaction and the "staying powers" of the unstable reacting elements; and the character of a complement largely depends on the way in which these factors are balanced. This balance is of particular importance in complement fixation tests. Owing to the nature of such tests, however, the balance is artificially disturbed. In the first part of the experiment the attempt is made to use up one factor ("staying powers" of reacting elements), and then the serum, modified to greater or less degree by this treatment, is tested for a function (hæmolytic activity) which depends for its laboratory demonstration on both factors. Hence arises the difficulty which is usually expressed by saying that susceptibility to fixation is not necessarily in proportion to hæmolytic capacity. In some cases the difficulty may not be of practical importance. For example, (1) a complement which loses its power (or is inactivated) slowly, but acts quickly, will not be easily fixed and will be a good hæmolysin: or (2) a complement which has feeble "staying powers" and is inactivated readily, but acts slowly, will be readily fixed and will be a poor hæmolysin. In these two instances the two factors harmonise, and therefore the hæmolytic titre coincides with capacity for fixation. But in other cases the two factors may pull in opposite directions, and then the actual result will vary according to the relative influence of each. For example, (3) a complement which is inactivated readily, but acts quickly, may be a good hæmolysin although readily fixed; and (4) a complement which is inactivated slowly, but acts slowly, will not be easily fixed, but may be a poor hæmolysin.

This is one very important example of purely artificial experimental requirements which affect the use of the hæmo-

lytic system as an indicator. In some of the articles which I have summarised above, another example emerges. Corpuscles together with heated immune serum form a compound with adsorptive capacities which vary according to the proportions in which the two substances are mixed. Active serum is particularly susceptible to the action of adsorbing reagents, and consequently the fate of complement is affected by the arbitrary proportions in which corpuscles and immune serum are mixed. This consideration forms part of the general problems which are discussed in the following sections on anticomplementary action.

Before proceeding to this subject it will be useful to take stock of the considerations which I have already put forward. They will not solve the difficulties about the theory of the Wassermann reaction, but they may help to clear up some of the confusion, particularly with reference to the function of complement.

Evidently one must abandon the idea that complement is a fixed chemical substance obeying the ordinary quantitative laws of chemical combination. It is no use trying to patch up this idea by supplementary hypotheses, *e.g.*, weaker and stronger combining affinities, multiplicity of complements, presence of "complementoids." The purely chemical and quantitative conception of the activities of complement, whether presented in a simple or in a complicated form, has failed and must be replaced by something else.

Complement is a property of fresh serum and depends on two characteristics of the serum, its adsorbability and its chemical activity, the former being probably less thermolabile than the latter.

In virtue of its colloidal state, fresh serum contains molecules which (1) are in unstable equilibrium, (2) tend to aggregate together, and (3) tend to form adsorption compounds when brought into contact with other colloids in a physically suitable medium.

In its chemical aspect, fresh serum contains molecules possessing atom groups with unsaturated affinities; many of these groups are unstable, uniting readily with groups attached to other molecules and readily severing their attachment to one molecule or another.

Complement resides in these elements which are unstable both from their colloidal and from their chemical nature, and in this respect is distinguishable from the more stable elements of serum such as immune body. But, in the present state of knowledge, which is admittedly imperfect, one cannot define complement more closely; it cannot be identified either with a particular colloid or with a particular chemical entity; it is a chemico-physical state of serum and, as such, is highly susceptible to physical or chemical influences.

One must remember that the physicists insist on the impossibility of drawing a sharp or ultimate distinction between colloids and crystalloids.

S. W. Young (1916), in the chapter on Colloids in Zinsser's "Infection and Resistance," writes as follows:—

"Current opinion seems to be leading rapidly to the general acceptance of the hypothesis that in liquid systems of two or more compounds we have to do with a continuous series of conditions ranging from coarse suspensions through suspensions of increasing fineness (increasing degrees of dispersion), to finally the molecular and ionic states of solution. The opinion is also growing that, although for certain practical purposes the classification of all such systems in one way or another, as in terms of the various degrees of dispersion, may be useful, the excessive use of such classifications is likely to narrow rather than broaden our conception of the whole subject matter of the field. It would seem that the most stimulating point of view is to be reached from the acceptance of the suggestion of Wolfgang Ostwald, that the chief problem of colloid chemistry at the present time lies in determining the influences of the degree of dispersion upon the physical and chemical properties of all liquid solutions, mixtures, suspensions, or what not."

Applying this consideration to complement, fresh serum is to be regarded as a complex and unstable mixture of (1) free ions, (2) free molecules with atom groupings ready to enter into new combinations or to dissociate, and (3) aggregates of molecules in various and variable degrees of dispersion. Complement may be defined as a function of (1), (2) and (3).

Specific Anticomplementary Action.

I propose to approach the subject from the laboratory standpoint, and to take the forensic test as an example.

When the complement fixation test is based on what is fully admitted to be a specific antigen-antibody reaction, as in the diagnosis of blood stains for forensic purposes, practical experience has shown that certain precautions must be very carefully observed. Sachs and Ritz are leading authorities on this subject, and have published (1913) a standard description of the technical requirements for the test.* For the purpose of the present report it will be useful to call attention to certain points to which they attach importance. I have selected the following, which, it is to be understood, are not intended to cover all the requirements for a complement fixation test:—

1. Complement is not a substance which can be demonstrated directly; it can only be recognised by its function of acting on cells with the aid of amboceptor.

2. It is recommended that small doses of the hæmolytic amboceptor should be employed.

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* Kolle and Wassermann's *Handbuch der pathogenen Mikro-organismen*, 2nd edition, Vol. III., p. 72.

3. Blood corpuscles and amboceptor should be added in the smallest volume possible, in order to avoid too great a difference between the volumes in the first and the second parts of the experiment. There are "antagonistic functions" (Bordet and Gay) which interfere with complement fixation, and these are more or less weakened by dilution.

4. Complement should be added immediately after antigen and antibody, because (Dean) fixation is weakened if antigen and antibody remain together for a long time before complement is added.

5. The three ingredients must remain together for sufficient time (1–1½ hours) before the hæmolytic system is added, as there are differences in the rapidity of the reaction with the same absolute capacity of fixing complement.

6. Complement fixation depends, often within narrow limits, on quantitative relations; excess of antigen and also excess of antiserum may inhibit it. So graded doses must be used to hit off the optimum. Even when the antibody is the substance to be tested, it is often found that graded doses of antigen bring out sharper differences between the strengths of antisera than gradation of antiserum.

7. The optimum of antigen and of antibody must be determined by experience for each form of the test. For each dose of antibody there is an optimum of antigen, and *vice versâ*. In practice, excess of antigen is a more serious mistake than excess of antibody. It must be remembered that a double dose of antigen may be less anticomplementary than a single dose.

8. In forensic tests for specific protein, the antisera must be specially titrated for complement fixation. The suitability of a serum for the precipitin test does not justify the assumption that it is suitable for complement fixation.

9. An antigen may be so altered as to fail to give the precipitin reaction whilst still giving complement fixation.

10. As regards the sensitiveness of the forensic test, complete fixation should be given with $\frac{1}{10,000}$ c.c. of the serum used as antigen, but not with $\frac{1}{1,000,000}$. Heterologous antigens must give complete lysis in doses as high as $\frac{1}{100}$ and $\frac{1}{1,000}$. The highest of the graded doses of antigen to be used should be that which gives a definite precipitin reaction.

11. For maximum sensitiveness in detecting antigen, a high titre antiserum should be used in as large an amount as possible without interfering with the reaction; for sharp differentiation, the use of less strong antisera in smaller doses is recommended.

The interpretation of the above principles depends on one's theories. For example, the Ehrlich school would interpret them very differently from the Bordet school, and, again, the followers of Friedemann would introduce explanations of their

own. I propose, to begin with, to suggest an explanation which is frankly one-sided and is purposely introduced to focus attention on one aspect of the problem.

The above rules may be regarded as being all directed to the same object, viz., the arrangement of a biological test (specific complement fixation) in such a way that it is not interfered with by the artificial conditions which occur *in vitro*. From this point of view I think that these rules, taken in order, may be interpreted as follows :—

(1) When a biological reaction takes place in the first part of the experiment, active serum loses a particular activity, having expended it upon sensitised antigen, just as one may imagine that the living plasma, when supplemented by the requisite immune body, expends some of its activity in acting upon a foreign antigen.

(2) Complement may fulfil its proper function in the first part of the test, but some remnants of its activity may be left ; and these remnants may react with an indicator made too sensitive by excess of hæmolytic immune body.

(3) *In vitro*, the activity and "staying powers" of the limited amount of active serum employed depend largely on its concentration, and this fact must be remembered in interpreting results.

(4) *In vivo*, the influence of the plasma begins to be exercised when the antigen-antibody combination is in the nascent state, and this condition should be reproduced, as far as possible, *in vitro*.

(5) The time required for digestion differs in amount under different physiological conditions of the digestive fluids.

(6) The efficiency of a digestive process obviously depends on the relative quantities of digestive fluid and material to be digested. Thus, heavily sensitised blood corpuscles require less complement for lysis. *See* last sentence of (2).

(7) Optimum quantities vary with the quality of the material to be digested.

(8) and (9) The using up of complement by a digestive process is not necessarily the same thing as the mere adsorption of complement in a specific precipitin reaction.

(10) and (11) When the biological properties of the reagents are not interfered with by the use of unsuitable proportions, the reaction may be made extremely sensitive, both quantitatively and qualitatively.

The above interpretation is open to the objection, which I fully recognise as important, that many authorities hold a different view as to the nature of so-called "biological

reactions" in the test tube. Such objection might be expressed as follows:—

Though complement fixation is a reaction in which biological products are employed, it is performed under conditions which are highly artificial, and it is not arranged for the purpose of reproducing any hypothetical function of complement in the living body. It is a test which is based on the principle that the antigen-antibody complex adsorbs complement; and all the details of technique are arranged so as to satisfy the colloidal, physical, and chemical conditions requisite for demonstrating this principle.* The question whether the adsorbed complement exerts any particular action upon antigen does not arise, as it is immaterial for the purpose of the test.

This latter view is attractive because it appears simple and practical. But further consideration is needed before it can be accepted as a complete explanation.

Action of Complement on Sensitised Antigen.

This question may be introduced by calling attention to some work by Toyosumi and Spät, which, though not directly planned for this purpose, relates to the interaction between complement and antigen when the latter is in the form of a specific extract.

Toyosumi (1909)† made some investigations on the part played by immune body in specific fixation of complement. He used anti-cholera serum, cholera vibrios, and extract of cholera vibrios. The last was prepared by heating a saline emulsion of vibrios for two hours at 60—65° C., then shaking for 24 hours at room temperature, and centrifuging until a clear fluid was obtained.

His serum gave the following titre:—

Serum + Vibrios.			Deposit + 0·1 Comp. (1 hour at 37° C.)	Supernatant Fluid + 0·1 Comp. + $\frac{2}{5}$ Loopful of Vibrios. (1 hour at 37° C.)
0·01	$\frac{2}{5}$ Loopful	½ hour at 37° C., then centrifuged.	Granules	Vibrios + a few granules.
0·005	„		„	„
0·001	„		„	„
0·0005	„		Granules + a few vibrios.	„
0·0001	„		Vibrios + a few granules.	„
0	„		„	„

The next protocol was advanced to show that, when vibrios were added to an incubated mixture of serum and extract, they removed the immune body up to full titre. This was regarded as evidence that none of the immune body had been fixed by extract.

* For example, antigen and antibody, as Col. Harrison has suggested to me, do not break up so as to form the maximum adsorbing surface if one of them is in excess; and, again, this maximum surface is obtained when the precipitate is in the invisible stage.

† *Centralbl. f. Bakteriol,* Orig. XLVIII., p. 325.

Serum +	Extract +	Vibrios.			+ Comp.	Result.
0·05	0·2	$\frac{2}{5}$ Loopful	1 hour at 37° C.	1 hour at 37° C., then centrifuged and washed.	0·1	Granules
0·01	0·2	,,			0·1	,,
0·005	0·2	,,			0·1	,,
0·001	0·2	,,			0·1	,,
0·0005	0·2	,,			0·1	$\frac{4}{5}$ granules + $\frac{1}{5}$ vibrios
0·01	0	,,			0·1	Granules
0	0·2	,,			0·1	Vibrios + a few granules.
0	0	,,			0·1	,,

It was also found that, when complement was included with the incubated mixture of serum and extract, the bactericidal immune body remained intact, although complement was fixed. Thus :—

Serum +	Extract +	Comp. +	Vibrios.			Deposit (examined at once).	Deposit + 0·1 Comp. (1 hour at 37° C.)
0·05	0·2	0·1	$\frac{2}{5}$ Loopful	1 hour at 37° C.	1 hour at 37° C., then centrifuged.	Vibrios	Granules
0·01	0·2	0·1	,,			,,	,,
0·005	0·2	0·1	,,			,,	,,
0·001	0·2	0·1	,,			,,	$\frac{3}{5}$ granules + $\frac{2}{5}$ vibrios.
0·0005	0·2	0·1	,,			,,	$\frac{1}{2}$ granules
0·01	0	0·1	,,			Granules	—
0	0·2	0·1	,,			Vibrios + a few granules.	Vibrios + a few granules.
0	0	0·1	,,			,,	,,

The following experiment was intended to show that "the "mixture of extract and immune serum, even after removal of "the bactericidal immune body, is fully competent to absorb "complement."

Serum +	Extract +	Vibrios.			Deposit + 0·1 Comp. (1 hour at 37° C.)	Supernatant Fluid + 0·1 Comp. (1 hour at 37° C.); then + Sensitised Vibrios (1 hour at 37° C.).
0·05	0·2	$\frac{2}{5}$ Loopful	$\frac{1}{2}$ hour at 37° C.	$\frac{1}{2}$ hour at 37° C., then centrifuged.	Granules	Vibrios + a few granules.
0·01	0·2	,,			,,	,,
0·005	0·2	,,			,,	Vibrios
0·001	0·2	,,			,,	,,
0·0005	0·2	,,			Granules + a few vibrios.	Many vibrios + a few granules.
0·0001	0·2	,,			,,	Many granules + a few vibrios.
0·01	0				Granules	Granules
0	0·2	,,			Vibrios + a few granules.	Granules + a few vibrios.
0	0	,,			,,	Granules

When this test was repeated with sensitised red cells (which were a more sensitive indicator than sensitised vibrios), it was shown, more clearly than in the above protocol, that the larger doses of immune body were less effective than the smaller. This, Toyosumi observed, was not due to a Neisser-Wechsberg phenomenon of complement deviation. With the stronger concentrations of serum, a precipitate was visible in the mixture of extract and serum; and this precipitate, which contained the complement fixing property, was removed by centrifuging. But with the weaker doses of serum no visible precipitate was formed, and the substance capable of fixing complement remained in suspension.

What was the substance in the immune serum which determined the fixation of complement? Toyosumi thought his experiments proved that neither the cytophil nor the complementophil group of the bactericidal amboceptor was concerned; they also showed that removal of agglutinins left complement fixation unaffected. The only process which weakened complement fixation was the removal of a visible precipitate, which itself fixed complement strongly, and must, therefore, contain the necessary components. His view, accordingly, was that the precipitins produced in the dissolved bacterial substance a modification which caused complement fixation. The bacterial substance which underwent this change might remain in the solution and need not necessarily come down as a precipitate.

It is interesting to note that bactericidal immune body was not damaged by union (or contact) with either specific extract or complement. As regards the last protocol, it may be noted that Toyosumi has not put up a control to show that the anticomplementary action of the supernatant fluid was due to a specific reaction, *e.g.*, a control, containing corresponding doses of inactivated normal serum in place of immune serum, which yielded supernatant fluid possessing no anticomplementary action.

Spät (1913)* investigated specific complement fixation with particular reference to the part played by antigen. The following is an example from his experiments with extract of cholera culture and cholera immune serum.

(1) Extract, immune serum, and complement were incubated for 1 hour at 37° C., and then heated at 56° C. for half an hour to inactivate free complement. (2) Fresh complement was then added, and, after 1 hour at 37° C., sensitised corpuscles. The result was complete lysis.

This was his main experiment. Controls showed that complete inhibition was produced when complement was omitted from the first part of the experiment, and that neither extract alone nor immune serum alone was auto-inhibitory.

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* *Biochem. Zeitschr.*, Vol. 56, p. 21.

Six Sets of Three Tubes and (VII.) Controls for Extract and Serum.

Extract.	Immune Serum.	Complement.	One hour at 37° C.; then ½ hour at 56° C.	Addition of 0·001 immune serum.	Addition of 0·1 extract.	Addition of 0·05 complement to each tube; after 1 hour at 37° C., addition of sensitised sheep corpuscles.	Results.
I. 0·25	0·075	—					Complete inhibition.
0·25	0·050	—					,, ,,
0·25	0·010	—					,, ,,
*II. 0·25	0·075	0·3					Complete lysis.
0·25	0·050	0·3					,, ,,
0·25	0·010	0·3					,, ,,
III. 0·25	0·075	0·3 (Inactivated.)					Complete inhibition.
0·25	0·050	0·3 (Inactivated.)					,, ,,
0·25	0·010	0·3 (Inactivated.)					,, ,,
IV. 0·25	0·075	0·3					Complete lysis.
0·25	0·050	0·3					,, ,,
0·25	0·010	0·3					,, ,,
V. 0·25	—	0·3					Complete inhibition.
0·25	—	0·3					,, ,,
0·25	—	0·3					,, ,,
VI. 0·15	0·075	0·3					,, ,,
0·15	0·050	0·3					,, ,,
0·15	0·010	0·3					,, ,,
VII. 0·25	—	—					Complete lysis.
—	0·075	—					,, ,,

* Main experiment, the others being controls.

Spät's interpretation of the first part of his experiment was :—

(*a*) Fresh complement was necessary because, when replaced by inactivated complement, complete inhibition was the result. Thus the inactivated complement did not "block" a hypothetical complementophil group of immune body. (Test III.)

(*b*) Absence of complement fixation was not due to want of immune body because, when further immune serum was added at the end of the first part of the experiment, the result was complete lysis, as before. (Test IV.)

(*c*) Presence of immune body was necessary because, when this was omitted from the first part of the experiment and was subsequently added, the result was complete inhibition. (Test V.)

(*d*) A change must have been produced in the extract because, when some of the extract was added after the first part of the experiment, hæmolysis failed to occur, *i.e.*, the new and unmodified portion of extract again made the binding of complement possible. (Test VI.)

The author regarded his observations as confirming the view of Bordet that the function of immune body was to "sensitise," like a mordant. This was in accord with previous work by Spät and Weil, showing that immune body did not unite with antigen and complement but could be recovered, after the experiment, qualitatively and quantitatively unaltered.

As a result of being sensitised, antigen was acted upon by complement; the result of this action was to produce a modification of antigen which was demonstrated in the hæmolytic experiment (loss of power to inhibit lysis).

Hence, in Spät's view, complement fixation was to be brought into line with agglutination, precipitation, and bacteriolysis, all of which were merely different ways of demonstrating the same essential reaction between antigen and antibody.

Spät's technique may be illustrated from the example quoted above.

He used graduated doses of immune serum, in this case 0·075, 0·05, and 0·01, and added to each tube 0·25 extract and 0·3 complement; after 1 hour at 37° C. and then ½ hour at 56° C., he added 0·05 complement and, after 1 hour at 37° C., sensitised sheep's corpuscles. Each tube gave complete lysis.

For the success of the experiment it was necessary to use the right quantities of complement. The amount required for the hæmolytic indicator, in this case 0·05, had to be determined each day by titration, owing to variations in complement. The amount of complement to be used with antigen and immune body had to be enough to exert its full powers of fixing antigen, but excess had to be avoided. If marked excess were used the experiment would fail, because lysis would take place in the controls which should show inhibition. If less than the optimum quantity were used the experiment might still be useful, owing to the safeguard of employing immune body in graduated doses. For example, in another experiment recorded, 0·3 of complement was insufficient for complete inhibition of anticomplementary power in the tube containing the extract most highly sensitised with immune body (0·075), and consequently the hæmolytic indicator showed incomplete lysis; but in the tubes containing less immune body (0·05 and 0·01) 0·3 of complement was sufficient, and both tubes gave complete lysis.

Theoretically, the correct amount of complement to use should be determined by preliminary trials with increasing quantities and application of the hæmolytic test to indicate the maximum amount used up by antigen and immune body; but Spät found that this took too long and did not leave enough time for the main experiment. So he preferred to start at once with the amount (0·3) which he thought, from previous experience, would probably be correct, and to take the risk of having to discard the experiments which failed.

In stating that excess of complement had to be avoided, Spät apparently means that a much larger quantity than 0·3 c.c. might damage extract, even in the absence of immune serum, and so give lysis. This error would be revealed in Test V.

As regards the danger of using too little complement, it is implied that the most strongly sensitised extract has the greatest anticomplementary power, and that it takes more complement to destroy this power than in the case of more weakly sensitised extract.

Comments on the Mechanism of Complement Fixation.

I now revert to the question as to the way in which complement is used up in a specific reaction. In some cases it is obvious that the process does not stop at adsorption. In a hæmolytic experiment the corpuscles do not merely adsorb complement but are visibly changed by it. Again, when complement is brought into contact with sensitised bacteria, there is evidence that the normal properties of the bacteria are impaired, and, as further evidence of loss of function, bacteriolysis may be produced. Coming to antigens in the form of extracts, Toyosumi shows that sensitised bacterial extract, after removal of immune body, is still anticomplementary, though he does not make it clear how far such interaction between complement and extract can be explained by adsorption alone. In relation to this question, the main interest of Spät's experiment is the evidence that, when complement, aided by immune body, is dealing with a specific extract, it behaves in the same way as when it is dealing with intact cells, *i.e.*, the extract, like the cells, is specifically modified. It is natural, on biological grounds, that this should be the case. Laboratory facts, therefore, support the general principle that complement fixation tests for specific protein involve modification of sensitised antigen by complement.

Here I have come in close proximity to a controversial issue. On the one side it is maintained that complement enters into chemical combination with antigen-antibody. The opposing view is that complement is simply adsorbed by the specific precipitate formed by the interaction of antigen with antibody, and that this adsorption takes place pre-eminently when the precipitate, being in the nascent state of formation, is in such fine particles that they are invisible to the naked eye.

In one aspect I do not think this controversy is of very great importance. The adsorptionist is obliged to concede that capacity for adsorption ultimately depends on chemical constitution, and that there is no sharp contrast between adsorption and chemical combination. And it would be futile for the chemist to ignore the importance of adsorption in these reactions. For these reasons it appears that the antagonism between the two theories need not be accentuated.

But, from another aspect, a question of considerable importance is involved. Are all substances anticomplementary in the same way? The adsorptionist theory would suggest that

they are. From this it would follow that the specific part of the reaction is at an end when interaction of antigen with antibody has occurred, and that the destruction of complement is merely a mechanical consequence of the formation of this adsorption complex, a consequence differing in no respect from the destruction of complement by a heterogeneous multitude of non-specific anticomplementary substances. There is, however, another view. According to this, all three elements are involved in the specificity of the reaction, which consists in the action of active serum, aided by immune body, upon antigen. Complement is "active" in the literal sense of the word; it becomes "inactivated" in the sense that it is used up when it has done its work in a biological reaction. This is specific "inactivation of complement."* Non-specific inactivation simply means that some physical, colloidal, or chemical reagent has made complement incapable of performing its normal biological function. The two processes of inactivation are thus essentially different, though adsorption plays an important part in each. For example, complement might be used up merely in its capacity for being adsorbed, as when there was an excess of adsorbing surface, or it might be exhausted partly by adsorption and partly by chemical action, as when adsorbing substance was less and its composition such as could be acted upon.

The difference between these two views is of practical interest. If laboratory technique were perfect, it might not be an urgent matter; one might be content to say that, if a particular rule of thumb is carefully observed, the result will be accurate, irrespective of all theories as to the way in which complement acts. But these theories at once become important when questions of improving technique are raised, *e.g.*, questions as to the order in which the reagents are to be mixed, the optimum quantities, the time factor, and the best temperature.† The answers to these questions are necessarily dependent on general principles governing the function of complement in a specific reaction.

Factors disturbing the Mechanism of Complement Fixation.

Normal Antilysins in Serum.

Normal serum sometimes has the effect of interfering with the complement fixation test.

One way of attempting to explain this is to fall back on the general idea that the peculiar properties of a complex substance

* The phrase has become stereotyped, and therefore must be used; but it is unfortunately chosen, since complement is really the active reagent which "inactivates" antigen.

† For example, it is a question whether ice-chest fixation favours only the adsorption part of the reaction.

such as serum are due to "antagonistic elements" which more or less balance each other under ordinary conditions but may, under special circumstances, become unbalanced and thereby bring their antagonism into prominence. Thus it is supposed that certain "anti-" substances are discoverable in serum and that these may neutralise complement.

This method of explanation deserves attention because it has assumed many forms and to some of these considerable importance is still attached. It will, therefore, be useful to consider how far, and in what form, the "antagonistic" idea is a really helpful explanation and how far there is danger of its leading into a blind alley. I begin with some of the simpler conceptions and postpone to a later section (pp. 154–63) the consideration of those ideas of "balanced antagonism" which are based on Friedemann's analytical methods.

Noguchi (1906),* discussing "the thermostable anticomple-
" mentary constituents of the blood," advanced the view that blood serum normally contained certain principles which inhibited lysis by interfering with the action of complement. In the case of most sera the action only appeared after heating to 56° C. or higher. The action was non-specific and directed against alien as well as against native complement. "It would
" appear as if the inactivation of serum at 56° C. or thereabouts
" were due to a partial liberation of the antilytic principle
" which just about suffices to neutralise the action of the
" native complement. As the temperature of the serum is
" raised, up to a certain point, more and more of the antilysin
" is set free until the serum comes to contain an overplus, in
" excess of the quantity neutralising its own complement,
" which is capable of interfering with the action of additional
" native or alien complements." Serum heated to 90° C. was less antilytic than serum heated to 70° C. This Noguchi thought might be due to the liberation from the serum of a second group of hæmolytic principles.

His antilysin was removed from serum by digestion with blood corpuscles, which thereby acquired greater resistance to serum hæmolysins. By treating blood serum and corpuscles with ether the antilysin could be extracted and dissolved in saline. The solution was very thermostable and was termed by Noguchi "protectin."

Zinsser and Johnson (1911)† distinguished between the heat-sensitive and the thermostable anticomplementary bodies in human serum. "There appear to be two distinct antihæmolytic
" bodies, therefore, which may occur in normal serum. One of
" these is found in sera after heating, and is relatively thermo-
" stable; the other develops in sera on standing and is destroyed
" at 54°—56° C." The action of the thermostable bodies depended upon "a direct anticomplementary mechanism." The

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* *Journ. Exper. Med.*, VIII., p. 726.
† *Journ. Exper. Med.*, XIII., p. 31.

rate of development of the thermolabile body varied considerably in different sera; it appeared as the complement disappeared. They thought it was not pre-existent, as it was too strongly anticomplementary, but that it was formed in serum during the period of preservation. This thermolabile body inhibited alien as well as homologous complement, but, unlike Noguchi's thermostable body, it could not be absorbed from serum by digestion with red cells, nor did it render the treated cells resistant to hæmolysis.

The above statements illustrate one form of the idea that normal "antilysins" are to be found in serum. But this view obviously required some modification or expansion, because it was soon found that the capacity of complement to unite with the specific antigen-antibody complex could be interfered with by albuminous substances other than inactivated serum. The following abstract shows how Noguchi and Bronfenbrenner proposed to explain this difficulty.

Influence of Inactive Serum and Egg-albumen.

Noguchi and Bronfenbrenner (1911)* observed that inactive serum and egg-white interfered with complement fixation. In their experiments they showed that these substances prevented complement fixation by (1) syphilitic serum and antigen and (2) by meningococcus extract and antimeningococcus horse serum.

"This disappearance of the complementary activity in the "syphilitic reaction, as well as in the true Bordet-Gengou "reaction, is a phenomenon which incidentally accompanies "the fixation of certain serum constituents, some of which "possess a complementary activity. The presence or absence "of the complementary property in these protein components "does not influence fixation. Whether the disappearance of "the complementary activity during the phenomonen of "so-called fixation is due to a mechanical precipitation of the "molecules through absorption or whether it is due to a physico-"chemical alteration of the active molecules is unknown. It "is more probable that a chemical interaction takes place in "the case of the syphilitic reaction."

They found that "fixability" (*i.e.*, "interfering property") was gradually diminished when the sera and egg-white were heated at temperatures over 56° C., and that it totally disappeared at 85° C.

"The fact that the fixation is not selectively directed towards "complement has a very important meaning for exact serology. "The one-sided accuracy as to the complementary unity is no "longer sufficient for quantitative work. Both the complemen-"tary and the volumetric unity of a serum serving as the "source of complement should be taken into consideration."

Attempts to remove Antilytic Substances.

The idea that "antilytic" substances were present in serum was followed up by the endeavour to remove them. Various attempts have

* *Journ. Exper. Med.*, XIII., p. 92.

been made to employ barium sulphate for the purpose of getting rid of the properties of serum which interfere with the complement fixation test. Some of these are worth quoting, but it does not seem clear, according to M. Stern, that this method can be employed without vitiating the serum for the purpose of the test to which it is to be submitted.

Wechselmann (1909)* endeavoured to get rid of "complementoids," which he thought interfered with the Wassermann reaction, by absorbing them with a 7 per cent. suspension of freshly precipitated barium sulphate. Taking 0·9 c.c. of inactivated syphilitic serum which gave a negative reaction, he shook it for one hour at 37° C. with 3 c c. of saline and 0·5 c.c. of the barium sulphate suspension and then centrifuged until the liquid was quite clear. After this treatment he obtained a positive result. 72 control sera, including specimens from psoriasis, erythema nodosum, and several quite fresh cases of primary syphilis, never inhibited complement after treatment with barium sulphate. On the other hand, 66 sera from syphilitics were all positive after this treatment, and the reaction was generally strengthened in the case of those which were only weakly inhibitory when tested in the ordinary way.

Noguchi and Bronfenbrenner (1911),† in an article on barium sulphate absorption and the serum diagnosis of syphilis, stated that, using the proportions of serum and barium sulphate recommended by Wechselmann, they found that this treatment removed certain serum constituents which interfered with the fixation of complement. The reaction was stronger with inactivated sera, but with unheated sera the increase in the intensity of the reaction was almost negligible.

When used in higher proportions, barium sulphate removed some of the syphilitic antibody, as well as any anticomplementary substances present, and, if the amount of the salt was large enough, it might remove it all. The antibodies adhered to the barium sulphate, but retained their activity when thus absorbed. The antibodies of some syphilitic sera were less sensitive to absorption than others. The addition of normal serum to syphilitic serum had the effect of reducing the removal of syphilitic antibodies by barium sulphate.

In other experiments they observed that barium sulphate could readily absorb hæmolytic amboceptor, but had "almost no anticomplementary action."

Wolff (1911)‡ observed that serum from post-mortem material, when tested by the Wassermann reaction in the ordinary way, gave about 50 per cent. of false positives; but by using Wechselmann's barium sulphate method he could remove the non-specific substances interfering with hæmolysis and leave behind the true syphilitic bodies. The statement of Noguchi and Bronfenbrenner, that large quantities of this salt also removed syphilitic antibodies, was not regarded by Wolff

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* *Zeitschr. f. Immunitätsforschung*, Orig. III., p. 525.
† *Journ. Exper. Med.*, XIII., p. 217.
‡ *Zeitschr. f. Immunitätsforsehung*, Orig. XI., p. 154.

as being of any practical importance, because the quantity requisite for his purpose was very much smaller. He therefore recommended the method as useful for removing non-specific inhibitory substances from sera obtained from the living body, though he did not accept Wechselmann's "complementoid" theory.

To prepare the barium sulphate he mixed 15 c.c. of 5 per cent. sodium sulphate with 10 c.c. of 5 per cent. barium chloride, an excess of sodium sulphate being necessary to ensure rapid precipitation. The barium sulphate precipitate was washed in the centrifuge with normal saline and then suspended in 25 c.c. of saline. Equal parts of this suspension and serum were mixed.

In contrast to genuine syphilitic sera, normal sera made positive by digestion with an organ emulsion were again made negative by barium sulphate treatment.

25 c.c. of a false positive serum was treated with the same quantity of barium sulphate suspension, and the salt was then filtered, washed, dried, and extracted with alcohol in a Soxhlet apparatus. On evaporating down to 15 c.c. he obtained a clear yellow fluid, which became turbid on adding saline. This material was found to be as good a Wassermann antigen (in the same dose) as his alcoholic extract of syphilitic liver.

Margarete Stern (1912)* objected to Wolff's work on the use of barium sulphate in the Wassermann test. Following his technique, and not using the larger quantities employed by Noguchi and Bronfenbrenner, she found that (1) only a small percentage of unspecific fixations produced by human post-mortem serum, or by rabbit serum, was nullified by barium sulphate, and (2) this same procedure also nullified part of the specific fixation proper to syphilitic sera.

Without entering into controversial details it must, perhaps, be admitted that Wolff's work has not been confirmed. It is interesting to note that he found his method brought out a distinction between syphilitic sera and "artificial positives."

Colloidal Conditions in Relation to Complement.

In the preceding pages (pp. 121–4), I have begun by considering the conception that "antilytic" substances are to be found in normal serum, and I have followed up a line of thought which may be regarded as a natural development of this idea. Now it is time to ask what it is going to lead up to. I think the answer is that this line of thought leads into a blind alley, and that a clue to the better intepretation of experimental data concerning the inhibitory action of normal sera is to be found in the work of the Bordet school. I therefore call attention to an article by Bordet and Gay, and then add a

* *Zeitschr. f Immunitätsforschung*, Orig. XIII., p. 688.

summary of some experiments by R. M. Walker, which appear to support Bordet's principles

Bordet and Gay (1908),* writing on the absorption of alexin and the antagonistic power of normal sera, have recorded the following experiment :—

——	Normal Saline.	Inactivated Serum.	Alexin.	Sensitised Corpuscles.
Tube A - -	0·6	—	0·05	0·3
Tube B - -	0·3	0·3	0·05	0·3

The inactivated serum was normal rabbit serum ; the alexin was fresh normal guinea-pig serum ; and the corpuscles were 10 per cent. moderately sensitised bovine corpuscles in saline suspension.

On incubation at 35° C. haemolysis was complete in A in half an hour ; in B the corpuscles were intact after 3 hours, though slight hæmolysis subsequently took place.

This inhibitory action of normal inactive serum was shown by subsequent experiments to vary in intensity with increase of the serum and decrease of saline.

The effect of increasing the sensitisation of the corpuscles was shown in the following experiment :—

——	Normal Saline.	Inactivated Serum.	Alexin.	Weakly Sensitised Corpuscles.	Strongly Sensitised Corpuscles.
Tube A - -	0·7	—	0·05	0·25	—
Tube B - -	0·4	0·3	0·05	0·25	—
Tube C - -	0·7	—	0·05	—	0·25
Tube D - -	0·4	0·3	0·05	—	0·25

The inactivated serum was normal rabbit serum ; the strength of the emulsions of corpuscles was 40 per cent. ; the weakly sensitised had been treated with 1 part sensitiser to 10 parts blood ; the strongly sensitised had received three times as much sensitiser.

On incubation at 35° C., hæmolysis was completed in C in 12 minutes ; in A in 35 minutes ; and in D in 1 hour ; B was not hæmolysed.

The inactivated serum did not produce its inhibitory effect by interfering with the sensitisation of the corpuscles or by making them refractory to lysis. This was shown in the following way. Tubes in which heated serum had prevented hæmolysis were centrifuged and decanted ; on the addition of saline containing a trace of alexin, hæmolysis was produced.

* *Ann. de l'Institut Pasteur*, XXII., p. 625.

Nor did the heated serum alter the alexin, since it was found that the addition of saline brought about hæmolysis in an inhibited mixture of heated serum, alexin and sensitised corpuscles.

Bordet and Gay explained the above data on the ground that alexin fixation was a phenomenon of molecular adhesion (adsorption). The union of sensitiser and red cells constituted a complex with greater adsorptive avidity for alexin than the normal red cell; but the heated normal serum kept the alexin in suspension in the medium and gave it a more stable equilibrium, thus preventing its precipitation on the sensitised corpuscles. Salt solution, on the other hand, made the equilibrium more unstable, and so allowed alexin to condense or precipitate more readily on the sensitised cell. Similarly, as Gengou had shown, a trace of serum would prevent the clumping and lysis of red cells by a suspension of barium sulphate. The serum caused a dissociation of the particles of barium sulphate and gave it a milky appearance. Sodium citrate had a similar effect on barium sulphate, and it was also found that the citrate, in suitable doses, protected red cells from hæmolytic sera by preventing the fixation of alexin on the cells.

Applying the above considerations to complement fixation tests, Bordet and Gengou pointed out the risks of error from using too much serum in the first part of the experiment and too much saline in the second. As an example they give:—(1) alexin, 0·1 c.c. + bacterial suspension, 0·3 c.c. + 0·3 or 0·5 c.c. of the serum to be tested for antibody, with subsequent addition of (2) 1 c.c. of a weak suspension of corpuscles in saline. Fixation of alexin on the bacteria would be opposed by the large proportion of serum in the mixture, whereas the addition of much saline with the corpuscles would remove the antagonistic influence on alexin, and enable even a trace of free alexin to produce hæmolysis.

R. M. Walker (1917),* in an article on the colloidal nature of the Wassermann reaction, showed that complement was not fixed after the preliminary addition of complement, inactivated serum, or a 10 per cent. solution of egg-white. In each test he used 0·6 c.c. of antigen and 0·05 c.c. of syphilitic serum. He first showed that this combination produced complete inactivation of the following doses of complement:—0·1, 0·12, 0·14, 0·16, 0·18, and 0·2 c.c. He then incubated serum and antigen for 1½ hours at 37° C. (1) with smaller doses of complement, (2) with inactivated guinea-pig serum, (3) with inactivated rabbit serum, and (4) with 10 per cent. egg-white solution; after this period, fresh complement was added to each tube. The results were:—

(1) Treatment with complement in doses of - - } 0·05, 0·08, 0·1, 0·12, 0·14, 0·16.

* *Journ. of Path. and Bact.*, XXI., p. 184.

Addition of complement in doses of - - -	0·05,	0·04,	0·04,	0·04,	0·04,	0·04.	
*Results - -	+	+	+	+	+	+	
(2) Treatment with heated G.P. serum in doses of - -	0·05,	0·08,	0·1,	0·12,	0·14,	0·16.	
Addition of complement in doses of - - -	0·05,	0·1,	0·12,	0·14,	0·16,	0·18.	
Results - -	+	+	+	+	+	+	
(3) Treatment with heated rabbit serum in doses of	0·1,	0·12,	0·14,	0·16,	0·18,	0·2.	
Addition of complement in doses of - - -	0·1,	0·12,	0·14.	0·16,	0·18,	0·2.	
Results - -	+	+	+	+	+	+	
(4) Treatment with egg-white in doses of - - -	0·1,	0·12,	0·14,	0·16,	0·18,	0·2.	
Addition of complement in doses of - - -	0·1,	0·12,	0·14,	0·16,	0·18,	0·2.	
Results - -	±	+	+	+	+	+	

Possibly Walker's first experiment, showing the effect of a previous dose of complement, may differ in principle from his other experiments, and may be explained on the same lines as Spät's experiments (pp. 117–9).

The discrepancy between Spät's results and Walker's must be noted. When the former used inactivated serum (Test III.) he obtained "complete" inhibition"; Walker, in corresponding experiments, obtained lysis.

Evidently further investigation is needed before the issues raised by Spät and Walker can be cleared up.

Comments on Bordet and Gay.

I think it is immediately obvious that the work of Bordet and Gay makes it unnecessary to consider in detail the efforts, mentioned above, to discover "anti" substances which will explain how normal inactivated serum may interfere with the interaction of complement with the specific antigen-antibody complex.

The simple explanation is that the heated serum tends to produce in the mixture a colloidal condition which prevents the labile elements of the fresh serum from coming into contact with the sensitised antigen. As dilution with normal saline counteracts this tendency, the description of heated serum and saline as antagonistic forces is a statement of fact. But the "anti" substances postulated above were merely hypothetical, and may be dismissed as an attempt, naturally unsuccessful, to

* + = Lysis

explain a colloidal phenomenon on principles of simple chemical combination.

It is a routine precaution in complement fixation tests to ascertain that fixation, as shown by the absence of hæmolysis, is not attributable to the anticomplementary action of antigen alone in the first part of the experiment.

The simplest explanation of such anticomplementary action is that antigen, if employed in excess, directly adsorbs the constituents of fresh serum on which the activity termed complement depends. This may not always be the whole of the explanation; for example, if the antigen consists of bacteria, the fresh serum may contain some natural antibacterial immune body which renders antigen more capable of adsorbing complement. But still the main fact of practical importance is that the fixation test is vitiated if the adsorption of complement takes place when the antigen has not been sensitised by the antibody which is sought for.

Indirect Anticomplementary Action.

Some investigators, however, are not content with the simple explanation that the test may be interfered with by a direct colloidal interaction between antigen and complement. They resort to more complicated theories, of which the following is an example, viz., the theory that normal serum may interfere with the fixation test owing to an influence termed "indirect anticomplementary action."

Ritz and Sachs (1917),* working on the inactivation of complement by bacteria, used 24 hours agar slant cultures of *prodigiosus* emulsified in 5 c.c. of saline. The dose of bacillary emulsion was incubated for one hour with the serum, in total volume of 1·1 (or 1·2) c.c., and 1 c.c. of sensitised corpuscles was then added. The following is given as a typical example, though the results were not always so sharply defined:—

Amount of Bacillary Emulsion.	Hæmolysis with descending Doses (*a*—*e*) of G.P. Serum.				
	a 0·2	*b* 0·1	*c* 0·05	*d* 0·025	*e* 0·0125
1	0	0	0	Slight tr.	0
0·5	0	0	Slight tr.	Mod.	Slight tr.
0·25	0	0	Mod.	Almost complete.	Tr.
0·15	0	Slight tr.	Marked	Complete	Marked
0·1	Slight tr.	Complete	Complete	,,	Almost complete.
0·05	Complete	,,	,,	,,	,,
0·025	,,	,,	,,	,,	,,
0·015	,,	,,	,,	,,	,,
0·01	,,	,,	,,	,,	,,
0	,,	,,	,,	,,	,,

* *Zeitschr. f. Immunitätsforschung*, Orig. XXVI., p. 483.

Thus there was more inactivation of complement when more complement was present.

The authors regarded these results as the expression of indirect anticomplementary action, *i.e.*, the bacilli brought about changes in the serum which depended on its concentration, and this altered condition of the serum led to inactivation of complement. This they explained on Friedemann's principle; the bacilli made the serum globulins anticomplementary, though not necessarily giving a definite precipitate. The change could not be defined chemically, but was to be regarded as due to physical influences which, when sufficiently intensive, led to a precipitation of globulins.

At the same time they recognised, after Hirschfield and Klinger, that another factor might be at work, viz., direct action (either adsorptive or of other nature) of the bacteria upon the complement. This explained why their results were not always identical with those shown in the preceding table. The following is an example:—

Amount of Bacillary Emulsion.	Hæmolysis with descending Doses (*a*—*d*) of G.P. Serum.			
	a 0·2	*b* 0·1	*c* 0·05	*d* 0·025
1	0	0	0	0
0·5	0	0	0	Slight tr.
0·25	0	0	Slight tr.	Slight
0·15	0	0	Tr.	,,
0·1	Slight tr.	Slight tr.	,,	Mod.
0·05	Complete	Mod.	Mod.	,,
0·025	,,	Complete	Almost complete.	Marked
0	,,	,,	Complete.	Complete

Here the upper part of the table showed increased anticomplementary action with increased amount of complement, explained as in the preceding example, whilst the lower part of the table showed the reverse. The authors assumed that with the smaller amounts of bacilli there was a direct action on complement. With these smaller quantities physical "blocking phenomena" might play a part, with the result that even a small excess of serum would suffice to deprive the bacteria of the physical property which exerted an anticomplementary action on the serum.

They found that the "indirect" anticomplementary action of bacteria was best demonstrated by *prodigiosus*; it was not so good with staphylococci, and there was only slight indication of it with other species of bacteria which they tried. They thought it depended on the physical condition of the bacillary emulsion. With *prodigiosus*, anticomplimentary action was obtained in the warm, but not, or only in very slight degree, at 0° C.

Complement inactivated by the "indirect" method was restored by adding third component; hence they assumed that the loss of the function of the serum termed "third component" depended on changes in the globulins, and that susceptibility to these changes could be abolished by heat or certain chemical reagents.

In view of their observations on indirect inactivation, they thought it was not always safe to assume, in experiments with antigen, antibody, and complement, that anticomplementary action would be abolished or reduced by simply increasing the amount of complement. The opposite might happen; and, though the *prodigiosus* phenomenon might be a rare event, a combination of the indirect with other modes of anticomplementary action might not be of infrequent occurrence. They found indications of this in testing Wassermann extracts for autoinhibitory properties.*

Comments on Ritz and Sachs.

On the principle that a complicated explanation should be avoided when a simpler one will suffice, it does not seem necessary to postulate indirect anticomplementary action through modification of the serum globulins.

The bacillary emulsion in the larger doses, which are much larger than would be used in fixation tests, adsorbs the serum, and adsorbs it better when the serum is fairly concentrated. The smaller doses of bacillary emulsion either fail to adsorb (protocol 1) or only adsorb slightly, and will not adsorb more than a small amount of the serum components (protocol 2). As colloidal reactions intimately depend on the concentration of the colloids concerned, there seems nothing particularly remarkable about the observation that with the strong adsorbing reagent the optimum was found with concentrated serum, but with the weak reagent adsorption was poor, whether the serum was relatively concentrated or dilute.

At the same time I recognise that Ritz and Sachs have not only recorded certain facts but have endeavoured to explain them. The alternative explanation which I have put forward needs amplifying, as it would not be satisfactory criticism to dismiss their work with the comment that their data are simply what one might expect in colloidal reactions. Some further discussion of such reactions is therefore called for.

* Blumenthal (1913) stated in the *Zeitschrift für Immunitätsforschung* (Orig., Vol. XVI., p. 347) that alcoholic extract of congenital syphilitic liver was often markedly anticomplementary, and was more anticomplementary when more complement was present. This paradoxical effect could also be produced by adding inactivated serum to smaller doses of complement. Individual extracts behaved differently towards individual complements, so it did not matter whether the titre of the complement was high or low. The moral was to titrate complement with extract + a dose of normal serum.

Colloidal chemists, even when working with much simpler compounds than those encountered in biological reactions, find that processes of molecular adhesion are so delicate and so liable to variation that they cannot be kept absolutely under experimental control. The remarks of S. W. Young on this point are worth quoting. "The one great difficulty which lies " in all such investigations is that it is a matter of very great " difficulty to duplicate results. The nature of any colloid " sol or gel depends so greatly upon its whole previous history, " apparently down to the least detail, that great discrepancies " in experimental results are found." The difficulty is much greater when dealing with complex bodies the chemical nature of which is to a large extent obscure, such as the constituents of sera, animal cells, or bacteria. Hence, for example, it is not unlikely that the two cultures of *prodigiosus* used in protocols 1 and 2 were not exactly the same in colloidal structure and properties, though prepared in exactly the same way. And it is equally possible that there was some minute colloidal difference, perhaps in molecular aggregation, between the sera used in protocols 1 and 2. One has always to be on the lookout for circumstances which are not under control.

Such circumstances, in immunity work involving colloidal reactions, are of two sorts, (1) artefacts or other disturbing factors incidental to experimental difficulties, and (2) natural variability of the colloids concerned. Obviously the endeavour is made to exclude (1) as far as possible, but it is equally important to distinguish between (1) and (2), and to recognise the impossibility of excluding (2), which is biological bed-rock.

In expansion of my last remark, I revert to the introduction of this report, where I recalled the well-recognised principle that the elementary data of metabolism and digestion are the foundation of immunity work. This principle must be supplemented by another conception which is also essential, viz., vital processes involve changing states of molecular adhesion. One may explain this roughly by saying that, just as in laboratory experiments these changing states are not always under complete control, though controlling forces are set up to deal with them, so in nature conditions of molecular adhesion are, as it were, policed by plenty of controlling forces and usually behave in the expected manner, but sometimes they momentarily evade the vigilance of the police, and then something happens which is not supposed to be according to law. One may remark parenthetically that these occasions may possibly be opportunities for mutation and evolution. They are really according to law, because instability of equilibrium is a characteristic of colloids, which have what might be called a natural right to exhibit this property.

Applying these considerations to research work, when dealing with a colloidal reagent which exhibits irregularities of behaviour, one has to ask whether the irregularities can or ought to be

avoided or whether they are the natural expression of a colloidal property.

The Significance of Split Products of Complement.

There is a very large output of literature, which I do not propose to summarise, on the so-called "mid-piece" and "end-piece" of complement. This work is an attempt to throw light on the nature of complement by splitting fresh serum into a globulin portion and a residual fraction, and then observing how the two behave as regards the function known as complement activity. Its significance may be briefly considered in relation to certain important conceptions about the nature of complement.

(1) The view is still very widely held that complement (or any particular complement, according to those who believe in a multiplicity of complements) is a single substance, though not yet isolated, which is present in fresh serum. This view receives no support from the results obtained by the analytical methods of splitting serum. With minor exceptions, which have not been substantiated, it has not been found possible to show that complement is present in only one fraction of the serum. When the serum is split, complement is split. The analytical method has done nothing to show that "complement" can be differentiated from whole serum, or "mid-piece" from the globulin fraction, or "end-piece" from the residual fraction.

(2) Then there is the view, more or less analogous to theories of fibrin formation, that complement is not itself a substance but is a function attributable to the interaction of two or more special substances which are present in the serum. "Mid-piece" and "end-piece" do not fit in very well with this idea, because they are usually regarded as existing in normal serum not in the form of distinct substances but as component parts of one substance, the splitting up of which is purely artificial. There is, however, an important theory, for which Friedemann is mainly responsible, that there exists in normal serum a "balanced antagonism" between the globulins and albumins. I have referred to this theory at considerable length in a later section (pp. 154–63). In so far as this idea may be applied to complement, the work on "mid-piece" and "end-piece" may be regarded as an endeavour to throw light on the interacting substances present in the globulin and in the albumin fractions, which are supposed to promote or restrain complement activity according to the way in which they are balanced.

(3) A third view is that complement activity is not attributable to one or more special substances contained in the serum, but is a chemico-physical function of the serum as a whole. The failure of the analytical method to differentiate

complement from active serum lends support to this view, though such support is probably unintentional, since observations on "mid-piece" and "end-piece" are generally based on the assumption that complement is a special substance (or substances) distinct from the serum as a whole.

(4) The activity of complement, *e.g.*, in lysing sensitised cells, depends on two processes, adsorption and chemical action. This principle is certainly demonstrable by successive application of "mid-piece" and "end-piece," though, of course, it is also demonstrable in other ways, which do not involve the splitting of the serum.

(5) The following summary of Herzfeld and Klinger's theory is taken from the *Bulletin de l'Institut Pasteur.** Complement, as employed for hæmolysis. consists of two parts: the one (mid-piece) is composed of globulins, the other (end-piece) of products of advanced disintegration in the albumins of the blood plasma. The globulins have a tendency to fix themselves on the corpuscles already sensitised, and thereby to "hypersensitize" them, thus facilitating the fixation of the disintegration products, the presence of which ultimately determines the dispersion of the albumins of the cell-membrane, and so causes its penetration. In this interaction the lipoids of the cell-membrane take part as well as the albumins. Every influence which tends to damage the globulins by stabilising them also tends to diminish the complementary action of the serum; such influences are heat, treatment at 37° C. with pure water or with water containing certain proportions of acid or alkali, the addition of citrates, oxalates, &c. Another way of producing anticomplementary effects is to precipitate out of the serum the globulins in question; this is the mode of action of bacteria, agar, cobra venom, and the reagents used in the Wassermann reaction.

Controversial Issues about Complement.

If one compares the present position of research on complement with the conditions which prevailed a few years ago, it is obvious that scientific controversy has led to valuable progress. I refer in particular to the removal of the confusion which existed when theories about a multiplicity of complements and complementoids held a prominent place. These views having now receded into the background, thanks to the work of the Bordet school, there has been no need to recapitulate them in this report.

But the subject is still too obscure to be cleared up by attempted reconciliation and compromise between current opinions. It is still in the controversial stage, and involves issues which must be fought out before a better understanding can be reached as to the nature of complement.

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* Vol. XVII., 1919, p. 39.

The situation has become more intricate than it was in the early days of the controversy between Ehrlich and Bordet. Then the issues were fairly simple and straightforward and were to be proved or disproved by the laboratory facts adduced on either side.

The theories in dispute were relatively simple, because both sides conceived complement to be a special substance or substances, and controversy was confined to its mode of action. But, at the present time, this assumption cannot be taken for granted ; there are at least two ways of regarding complement ; it may be a function of the serum constituents as a whole, or, though its activity is influenced by its relation to other serum constituents, it may be a separate constituent. Until the issue is decided between these conflicting views as to the essential nature of complement, further problems as to the principles which govern its activity must necessarily remain involved in difficulties.

Again, in the Ehrlich *v.* Bordet controversies the laboratory data adduced by either side were within reasonably small compass, so that it was possible to repeat each experiment published by an opponent and to criticise it on laboratory grounds. This was obviously of great advantage in promoting the controversy to a useful termination ; but at the present time it is impossible to attempt laboratory analysis of all the evidence put forward in support of a particular theory, because the amount of literature on complement is overwhelming, and no investigator can repeat more than a small fraction of the experiments which have been published. This is a difficulty which is not confined to complement, but extends to almost all branches of immunity work. The total output is much too large to be assimilated ; individual workers on the same subject adopt different hypotheses, follow different methods, and employ different standards of criticism ; their results are confusing, and it is almost impossible to elucidate general principles out of a chaos of conflicting data.

Therefore, in dealing with complement at the present day, as contrasted with the early days of controversy on this subject, one cannot anticipate that patient analysis of laboratory data alone will suffice to clear away the confusion. One needs some method of sorting out the data which are likely to be useful, and of reducing to a minimum the wastage of energy caused by following up blind alleys.

I think the only way is to focus attention on the main issues about the nature of complement which call for settlement at the present day, and to group round each of these its appropriate laboratory data, the general object in view being to arrive at a satisfactory definition of complement.

There are many ways of raising crucial questions as to the nature of complement. The following is suggested as an example :—

Antigens employed in test-tube experiments with complement are of different kinds. Take (1) red corpuscles, (2) bacteria susceptible to lysis, (3) bacteria insusceptible to lysis, (4) bacterial extracts, (5) sera, and (6) syphilitic "antigen"; each of (1)–(5) is treated with its appropriate antiserum and (6) with syphilitic serum. Is the action of complement on each essentially the same? If not, what distinctions are to be drawn? Are (3)–(6), where there is no visible lysis, to be classed together and distinguished from (1) and (2)? If this is not a satisfactory division, what distinctions are to be drawn between (3)–(6)? Can it be said that in certain of these cases the activity of complement is the same as that whereby lysis was produced in (1) and (2), and that, in certain others, the only action which has occurred is adsorption, by the antigen, of the euglobulin fraction of the guinea-pig serum? Then there are further questions as to the differences, if any, between anticomplementary action in cases (1)-(6) and the admittedly non-specific anticomplementary action which a large variety of substances exert.

It would be helpful if each investigator who writes on complement, whatever branch of this subject he may select, would define his working hypothesis as to the nature of complement and show what support this receives from the results of his inquiry.

Part II.

WASSERMANN SUBSTANCE.

As nobody, so far, has been able to demonstrate the exact nature of "Wassermann substance," discussion is limited to the consideration of possible clues which may help to its identification at some future date.

Such clues may be found in investigations which have been specially directed to the search for them; they may also crop up incidentally in the course of research which is primarily directed to some other problem, such as the improvement of some detail in laboratory technique. Clues of the latter origin are difficult to pick out and group together, because they are often no more than side-issues. If one picked out the side-issue and omitted the main problem with which the author was concerned, it would distort his work; on the other hand, if such an article were summarised in a way satisfactory to the author, the summary might contain matter irrelevant to the particular clue to which one wished to call attention. When this dilemma has presented itself to me, I have chosen the latter alternative as being the less objectionable.

Differences in Syphilitic Sera.

Perhaps the first possibility to consider is that this mysterious Wassermann substance may not always be one and the same. Even if it is analogous to a genuine immune body, one must recognise that immune bodies are not always precisely the same, though produced in the same species of animal by the same (bacterial or other) species of antigen. Moreover, the differences between syphilitic serum and typically specific immune sera suggest that the Wassermann substance may be less sharply defined than a typical antibody. It is quite possible, therefore, that it may vary qualitatively, as well as quantitatively, in different persons and in different stages of the disease.

Such qualitative differences, if they exist, may partly explain why different antigens sometimes give different results, since the quality of a particular serum may be more suited to one antigen than to another. The following articles are quoted with particular reference to this point, though the collateral issue as to qualitative differences in antigens is necessarily involved.

H. Sachs (1917),* in an article on the influence of cholesterin upon the sensitiveness of organ-extract, showed that the addition of cholesterin not only enabled a serum to give a positive reaction with a smaller quantity of extract, but also enabled a higher dilution of the same serum to give a positive reaction. This, he remarked, was to be expected, owing to the frequently observed fact that syphilitic sera, giving a negative with some simple alcoholic extracts, gave a positive when the same antigens were reinforced with cholesterin. (He recognised that this was not universally the case, since some antigens were found which were not increased in their efficacy by the addition of cholesterin.)

He stated that his evidence was based on a large number of experiments in which he used alcoholic extracts, sometimes of ox heart and sometimes of syphilitic liver.

The following is the example which he has recorded of an experiment with alcoholic extract (ox heart) and the same reinforced with 4·35 c.c. of 1 per cent. alcoholic cholesterin solution to 100 c.c. of crude extract. He used (1) descending does of diluted extract with 0·025 inactivated patient's serum and 0·025 guinea-pig serum (total volume 0·75 c.c.), and (2) descending doses of diluted patient's serum with 0·25 of extract, diluted 6 to 10 times, and 0·025 guinea-pig serum (total volume, 0·75 c.c.). After digestion for an hour in the incubator, he added 0·5 c c. of a mixture (equal parts) of sheep corpuscles and diluted amboceptor. The extract, in double (0·5 c.c. of 1 : 6) the largest dose used, was not, or only very slightly, autoinhibitory.

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* *Zeitschr. f. Immunitätsforschung*, Orig. XXXI., p. 451.

TABLE showing hæmolysis obtained with 10 syphilitic sera (*a*—*k*). o = none; Spch. = slight trace; Sp. = trace; w = slight; m = moderate; st. = marked; f.k. = almost complete; k = complete. The effect of antigen without cholesterin (left half of table) is compared with the effect of the same antigen reinforced by cholesterin (right half). Gradations of extract (upper half of table) are compared with gradations of serum (lower half).

—	Antigen without Cholesterin.										Antigen with Cholesterin.									
	a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*k*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*k*
Amount of Extr. (1 : 6):																				
0·25 c.c. -	o	o	o	o	o	o	o	o	o	k	o	o	o	o	o	o	o	o	o	o
0·15 c.c. -	o	w	o	o	o	o	o	o	w	k	o	o	o	o	o	o	o	o	o	o
0·1 c.c. -	Sp.	f.k.	o	m	o	o	o	o	m	k	o	o	o	o	o	o	o	o	o	o
0·05 c.c. -	k	k	m	k	w	w	o	o	f.k.	k	o	o	o	o	o	o	o	o	o	Spch.
0·03 c.c. -	k	k	k	k	k	m	w	o	k	k	m	f.k.	Sp.	m	o	Spch.	o	o	w	f.k.
0·02 c.c. -	k	k	k	k	k	st.	m	Sp.	k	k	k	k	k	f.k.	w	Sp.	o	Spch.	m	k
0 c.c. - -	k	k	k	k	k	k	k	k	k	k	k	k	k	k	k	k	k	k	k	k
Amount of serum (1 : 10):																				
0·25 c.c. -	o	o	o	o	o	o	o	o	o	k	o	o	o	o	o	o	o	o	o	o
0·15 c.c. -	Spch.	m	o	Spch.	o	o	o	o	w	k	o	o	o	o	o	o	o	o	o	o
0·1 c.c. -	Spch.	k	o	Sp.	Spch.	o	o	o	f.k.	k	o	o	o	o	o	o	o	o	o	o
0·05 c.c. -	k	k	o	k	k	f.k.	Sp.	Spch.	k	k	f.k.	k	o	o	o	o	o	o	w	k
0·03 c.c. -	k	k	w	k	k	k	f.k.	m	k	k	k	k	o	Sp.	o	Sp.	Spch.	o	k	k
0·02 c.c. -	k	k	k	k	k	k	k	k	k	k	k	k	o	m	w	m	Sp.	o	k	k
0 c.c. - -	k	k	k	k	k	k	k	k	k	k	k	k	k	k	k	k	k	k	k	k

Apart from the evidence that cholesterin generally intensified the reaction, Sachs noted that the influence of this substance was not the same with all sera, but was more marked with some sera than with others. Sometimes sera which gave equal reactions with the plain extract were found to be of unequal strength when tested with the same extract reinforced by cholesterin; and sera which were unequal with the plain extract were not always unequal in the same proportion with the cholesterinised extract. Occasionally the inequality might be reversed, though such results were uncommon. Sachs attributed them to non-characteristic secondary factors, such as degree of alkalinity or acidity, which influenced the physical properties of the serum. At all events there were indications that, in their capacity for giving a Wassermann reaction, individual sera differed from each other not merely quantitatively, but also, to a certain degree; qualitatively.

Sachs' conception was that the properties which made a serum give a positive Wassermann reaction depended on the mixture of lipoids, or lipoid-protein combinations, which it contained. Anticomplementary activity came about through an interaction, under optimum conditions, between these lipoids and the mixture of lipoids contained in the extract. The optimum was attained when the lipoids of serum and extract were exactly suited for interaction, and the conditions for attaining it would vary according to the individual composition of each group of lipoids. This conception Sachs regarded as supplementing, and not as inconsistent with, the view that changes in the serum-globulins played an important part in the reaction.

It followed from the above considerations that the amount of cholesterin to be added to an extract must not be fixed arbitrarily, but must be determined by experiment with each extract employed. The optimum, which should not be exceeded, would depend on the concentration of the other ingredients in the extract, and would be higher for concentrated than for weak extracts. To determine the amount of cholesterin required, Sachs added different quantities to his alcoholic extract, diluted and undiluted, and tested each mixture on a large number of sera. With different extracts which he had employed, he had found that the optimum amount of cholesterin varied between 0·02, 0·16 and a maximum of 0·2 per cent.; usually it varied from 0·04 to 0·1 per cent. Occasionally the raw extract was found to contain the optimum.

As regards the part played by the serum, he found that cholesterin strengthened the reaction in many late stages of the disease and in latent cases, but in many early cases he obtained better results with Lesser's ether extract taken up with normal saline.

Though grading serum often extended the positive zone more widely than grading extract, he preferred the latter method,

because grading the former brought into greater play variations in the secondary and non-characteristic contents of the serum.

Sachs thinks the differences demonstrated in syphilitic sera are due to "secondary factors," but he has not shown that such factors are separable from "Wassermann substance," *i.e.*, he has not disproved qualitative differences in "Wassermann substance."

Mandelbaum (1918)* recently suggested that the difficulty arising from autoinhibitory sera might be overcome by diluting the sera (0·5 c.c. of serum to 2 c.c. of saline) before inactivating. It was important that the sera should be fresh. In his experience, all sera which were positive by the ordinary method were also positive when this modification was adopted, and, in addition, this modification gave more positives than the ordinary method with sera which were undoubtedly syphilitic.

With his technique only $2\frac{1}{2}$ doses of amboceptor were used, and autoinhibition was often encountered before this modification was adopted. Other advantages claimed for his modification were that readings could be taken in an hour, and that extracts which were inferior to the best gave unequivocal results. In other respects he strictly followed Wassermann's method.

This is introductory to the observations of Selter.

Selter (1918)† compared Mandelbaum's modification with Wassermann's method on 250 sera, tested simultaneously with the same reagents. The methods agreed in 203 cases, 160 being negative, 41 positive, and 2 doubtful. With the Wassermann method alone there were 16 doubtfuls, and with Mandelbaum's method alone there were 11 doubtfuls One case, in which the clinical condition was not known, was positive by Wassermann but negative by Mandelbaum. One case, reported clinically as not syphilis, was positive by Mandelbaum but negative by Wassermann. Of 18 cases, all clinically syphilitic, 2 were positive by Wassermann but negative by Mandelbaum, and 16 were positive by Mandelbaum but negative by Wassermann.

For each test, two antigens were used, and it was often found that the two did not give concordant results. With reference to this discrepancy, Selter stated that he had done 8,655 tests with two antigens obtained from the Wassermann Institute; discrepancies were found in 617, one giving a positive and the other a negative in 247 cases, one a positive and the other a doubtful in 166 cases, and one a doubtful and the other a negative in 204 cases. He remarked that, if one laboratory had based its reports on one of these antigens and a second laboratory on the other antigen, there would have been about 7 per cent. of discordant results.

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* *Münch. med. Wochenschr.*, p. 294.
† *Münch med. Wochenschr.*, p. 788.

It occurred to Freund (1918)* that, as Altmann had found that antiforminised typhoid bacilli retained their antigenic property in complement fixation reactions, antiformin might be useful for preparing the Wassermann antigen. Antiformin dissolved the organic material of the tissues without destroying the lipoids, which were the essential part of the Wassermann antigen; so he thought this reagent would be a useful means of obtaining the lipoids, and that antigens prepared in this way would be uniform in character. This would have the great advantage of eliminating the difficulties caused by the variability of antigens prepared in the ordinary way.

Experiments were made with antiformin antigens prepared from (1) the liver of a syphilitic foetus, (2) ox heart, and (3) ox liver. The organ, freed from connective tissue, was pounded in a mortar and made up with distilled water (1 grm. to 100 c.c.); the emulsion was filtered through gauze, and 5 c.c. of antiformin was added to 100 c.c.; the mixture was incubated for 1½ hours, and was then perfectly transparent. It was neutralised with 2 per cent. sulphuric acid, and the free chlorine was got rid of with sodium sulphite, starch and potassium iodide paper being used as indicator; 0·5 per cent. carbolic acid was added, and the antigen was kept in the ice chest.

A further method of treatment was also tried; 100 c.c. of antiformin antigen was treated with 40 c.c. of chloroform at 37° C. for 3 hours, the mixture being shaken for 5 minutes every half-hour. Then the layer of chloroform was separated by centrifuging, the remainder was evaporated on a water-bath, and the residue was taken up with 40 c.c. of 96 per cent. alcohol.

The autoinhibitory dose of the antiformin antigen was usually the same as that of the alcoholic extract of a normal organ; the hæmolytic dose was often found to be smaller.

A comparison between the antiformin extracts and an alcoholic extract of ox heart gave the following results:—

673 *Syphilitic Sera.*

	Both +	in 72·4	per cent.
Antiformin +, alcohol −	„	11·6	„
„ more strongly +	„	8·1	„
„ −, alcohol +	„	2·1	„
„ less strongly +	„	5·8	„

127 *Non-syphilitic Sera.*

	Both −	in 99·2	per cent.
Antiformin +, alcohol −	„	0·8	„ (one case).

In the tests, the total volume was 1·25 c.c., each reagent being made up to 0·25, Serum, 0·05; amboceptor, 2½ doses; sheep's corpuscles, 0·25 of 5 per cent. suspension.

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* *Deutsch. med. Wochenschr.*, p. 432.

The work of Selter and Freund suggests that differences in the reacting properties of syphilitic sera may be brought out by dilution of patient's serum before inactivation and by the use of different antigens.

In the course of a long article on the Wassermann reaction, Berczeller (1917) has made the following observations* :—

Influence of prolonged Incubation on Final Reading.—He took readings first at the end of one hour, and then, with continued incubation (in the thermostat at 37° C.) and frequent shaking, up to the end of eight hours. Usually, most of the reaction took place in the first hour, small changes were noted in the second hour, but not much alteration occurred afterwards. In some cases the effect of continued incubation was to weaken the reaction ; this happened less frequently with strong positives than with weaker positives or border-line cases ; the weaker the original reading, the more often was there a marked reduction in the final reading. He thought this was because complement entered into firmer combination when there was more reacting substance present in the serum, and that its combination was more reversible when the serum was weaker. This suggested that the interaction was in the nature of an adsorption process. Such processes were often imperfectly reversible, and the degree of reversibility depended on the concentration of the reacting substances.

The above observations were based on tests with inactivated serum. When active serum was used, he found cases where a strong positive was turned into a negative in 1—1½ hours, and also cases where a ± remained unchanged on continued incubation.

Function of Complement.—He regarded complement, and not patient's serum, as playing the part of amboceptor, because complement was modified by both serum and extract, and the modification exercised by the one was distinct from that exercised by the other.

Influence of Quantity of Serum.—Inhibition of hæmolysis did not always run parallel with the quantity of serum used. Amongst 68 positive sera tested in the active state, he found 14 which gave complete lysis in the stronger concentration but inhibited in the weaker, and 11 others which were less inhibitory in the strong than in the weak concentration. Amongst 38 inactivated positive sera, three completely lysed in the higher concentration and three were more lytic in the higher concentration than in the lower. He thought these results were attributable to Manwaring's "third substances" (substances present in the serum in addition to the recognised reacting bodies), and that alterations of concentration beyond certain limits might cause these substances to exert a profound influence.

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* *Biochem. Zeitschr.*, Vol. 83, p. 315.

Relative Advantages of grading Antigen, Complement, and Serum.—He found grading complement the least sensitive of the three methods. The reason, he considered, was that the quantity of complement fixed depended on the concentration, and in high dilutions of complement relatively less of it was fixed than in more concentrated. This was in accordance with observations that the binding of complement was partially reversible, whereas fixation of antigen or serum was not reversible.

Comparing gradation of antigen with gradation of serum, he found the latter much more sensitive. For example, it might be found that the amount of antigen sufficient to give inhibition was 20 times less with a positive than with a negative serum; but one unit of positive serum was often as potent as 250 or even 500 units of negative serum. This suggested to him that the serum reacted in a catalytic manner rather than by simple chemical combination. But grading of serum, though the most sensitive, was also the most complicated method, because it involved gradations not only of any Wassermann substance present, but also of the mysterious "third substances" or properties which might influence the reaction.

Anticomplementary Action of Serum.—Berczeller investigated the anticomplementary action of inactivated human sera without extract, and found that, as a rule, positive sera reacted more strongly than negative. But this reaction, in his experience, was much less specific than the Wassermann test, and he doubted if the relation of the one to the other was clearly demonstrated. In his opinion, the fact that sera alone could inhibit the very minute amounts of complement used in these tests gave rise to the question whether there might not be some antigen present in the serum which played a part in the reaction. This appeared to be the case. Neisser and Bruck stated that they had obtained active watery extracts from syphilitic blood. As watery antigens had fallen into disuse, Berczeller prepared antigens with alcoholic extracts of human sera, and compared the products made with positive and negative sera. He found the potency of different extracts quite irregular; sometimes positive and sometimes negative sera gave better extracts. Since antigen was demonstrable in sera and the amount present was variable, getting rid of the extract used as antigen in the Wassermann reaction, as proposed by Hesse (*see* pp. 145–6), would not be an advantage, but rather a disadvantage; the result of the test would become dependent on a new variable. Whilst recognising the importance of the difference between syphilitic and normal sera as regards antigen content, he regarded this as a disturbing factor, which did not suffice to explain the difference between the anticomplementary properties of syphilitic and normal sera.

Comments.

The interesting features in the preceding articles may, for the present purpose, be summarised as follows:—

Sachs finds that the use of different antigens demonstrates differences in syphilitic sera. He thinks these differences are due to "secondary factors."

Selter, using both the ordinary technique and Mandelbaum's modification, also finds that different antigens bring out differences in the response of syphilitic sera to the Wassermann test.

Freund's results point to the same conclusion.

Berczeller finds that different syphilitic sera may affect differently the final reading after prolonged incubation, and that different syphilitic sera are affected differently by alterations in concentration. He explains the first observation as due to differences in the firmness of the combination effected by strong and weak sera, and the second as due to the intervention of "third substances." He also finds that the presence of anticomplementary substance (? antigen) in syphilitic sera is a variable factor.

It is to be noted that the above authors have not succeeded in explaining these differences as due entirely to quantitative differences in the strength of syphilitic sera. Sachs and Berczeller, whilst not admitting that there may be qualitative differences in true "Wassermann substance," have to postulate "secondary factors" or "third substances." But as "Wassermann substance" has not yet been isolated, there remains open the alternative explanation that these so-called disturbing elements are really an essential part or function of syphilitic serum.

Leaving out of the question the peculiarities of such sera in the active state, which only make the matter more complicated, there do appear to be subtle differences in inactivated syphilitic sera which are not merely differences in "strength." Perhaps this is to be expected, as the abnormal products in the circulation of the syphilitic are not likely to be identical in all stages of the disease. These differences between one syphilitic serum and another, like the bigger differences between syphilitic and normal sera, probably depend on (1) chemical constitution of the abnormal constituents in the serum, and on (2) induced changes affecting the serum as a whole, in the condition of molecular adhesion. It is a difficult problem, at present unsolved, to decide how far the reaction between syphilitic serum and antigen depends on (1), and how far it depends on (2).

Direct Interaction between Syphilitic Serum and Complement.

I have already mentioned Berczeller's view (p. 142) that there is some direct action between syphilitic serum and

complement. His way of putting it is to say that complement plays the part of amboceptor between syphilitic serum and extract.

It is also to be noted (*see* Hecht, p. 165) that, when the three ingredients in the first part of the test are taken in pairs and the third is not added until the pair has been incubated, the strongest fixation is obtained when the selected pair is syphilitic serum and complement. This, again, suggests that the syphilitic serum alone may take something out of the complement.

I now quote Max Hesse's contribution to this line of thought.

Max Hesse (1916)* agreed with other observers that interference with the Wasserman test by autoinhibitory action of the patient's serum was uncommon; but occasionally it occurred with unexpected frequency in a particular series of tests, and then he noticed that the complement was not quite fresh or that the amboceptor was rather poor. So it occurred to him that possibly a slight readjustment in the balance of the reagents used in the reaction might cause this autoinhibition to appear more readily and more frequently, and that then the question might arise whether it was not a constant property of syphilitic sera, though usually masked when the Wassermann test was conducted in the ordinary way. He therefore determined to try the effect of abolishing all excess of both complement and amboceptor, and inactivated the serum for one hour in a water-bath to make sure of getting rid of all complement from it, as the varying amount of complement in active patient's serum would be a disturbing factor.

Working by the drop method and using minimal doses of complement and amboceptor with graduated doses of patient's serum, he found that the following arrangement of quantities gave useful results:—

——	Tube 1.	Tube 2.	Tube 3.	Tube 4.
Complement - - -	1 drop	1 drop	1 drop	1 drop
Patient's serum - -	3 drops	4 drops	5 drops	6 drops
Saline - - - -	3 drops + 1 c.c.	2 drops + 1 c.c.	1 drop + 1 c.c.	1 c.c.

After one hour at 37° C., addition to each tube of one drop 10 per cent. sheep's corpuscles and one drop amboceptor.

* *Wien. klin. Wochenschr.*, p. 605.

Example of Five Tests.

——	Wassermann Test.	Tube 1.	Tube 2.	Tube 3.	Tube 4.
1. Secondary syphilis -	+	+	+	+	+
2. Erythema multiforme.	0	0	0	0	0
3. Gumma nasi - -	+ +	+ +	+ +	+ +	+ +
4. Gonorrhœa - -	0	0	0	0	0
5. „ - -	0	0	0	0	0

In none of these cases was the serum autoinhibitory in the Wassermann test.

Every experiment conducted by this method required precise titration of complement and amboceptor, and a control to demonstrate that the patient's serum was completely devoid of complement.

He compared this method with the Wassermann reaction on the sera of 160 patients who were definitely either syphilitic or non-syphilitic, only three of the sera being autoinhibitory in the Wassermann test. In 132 cases the two methods gave corresponding results; five, all manifest syphilitics, gave a positive Wassermann, but were negative to Hesse's method; four, one case of syphilis and three of gonorrhœa, gave a negative Wassermann, but were positive to Hesse's method.

In 19 cases the latter method was unsatisfactory owing to the presence of normal hæmolysin for sheep's blood. In such cases hæmolysis occurred very rapidly and was first seen in the tube containing six drops of patient's serum. In order not to overlook this disturbing factor the tubes must be examined every five minutes.

This difficulty was engaging the author's attention, but he had not yet been able to overcome it. Getting rid of normal hæmolysin by digesting with sheep's corpuscles produced in the sera autoinhibitory properties which were shared alike by syphilitic and non-syphilitic sera. Attempts to produce a satisfactory hæmolytic system with human corpuscles failed owing to the production in the rabbit serum of agglutinins and precipitins as well as hæmolysins.

Whilst recognising that he had not proved his method to be a specific test for syphilis, Hesse thought that it deserved further attention, and that it might ultimately lead to a satisfactory means of simplifying the Wassermann reaction by getting rid of the antigen.*

* Dreyer and Ainley Walker (1913) stated in the *Journal of State Medicine*, vol. 21, p. 761, that "if we employ in an otherwise typical "Wassermann test reaction only a very weak hæmolytic system (much "weaker than is usually employed for the purpose), the reaction can quite "readily be obtained *without the use of any antigen at all.*"

His research incidentally led him to consider the question whether syphilitic serum, which he showed to be anticomplementary, contained less complement than normal serum. His experimental work failed to give concordant results on this point, possibly owing to the lack of identity between guinea-pig and human complement, or because experiments *in vitro* were not an accurate counterpart of what took place *in vivo*.

Comments.

In Hesse's tests the amounts of both complement and amboceptor are reduced to the bare minimum, so that very little loss or deterioration of complement will be followed by absence of lysis.

How is this loss or deterioration caused? As a rule, syphilitic sera are not markedly anticomplementary alone, when tested in the ordinary way with excess of complement for the hæmolytic reaction. But there may be a slight interaction with complement which, under these conditions, the hæmolytic indicator fails to reveal. According to one view, which I have outlined above (pp. 82–5), complement, when suitable conditions are presented to it, will partially reproduce in the test-tube the activity of plasma in the living body, *i.e.*, it will attempt to break down foreign material. When this material is the abnormal contents of syphilitic serum, already partially assimilated in the living body, one may imagine that the attempt is made but proves abortive. There may be slight interaction between the syphilitic serum and the labile elements of the fresh serum, and then the latter find no more work to do. This slight interaction may just turn the scale in Hesse's way of conducting the test with a bare minimum of complement.

If this assumption be correct, the interaction will be due to something abnormal in the syphilitic serum, but there is no evidence that this "something" is identical with the active substances in syphilitic "antigens." So it may, perhaps, be inaccurate to assume that the reaction is caused by the presence of antigen in the patient's serum.

As I remarked in discussing the relation of syphilitic serum to antigen, there are always two factors in the serum which have to be considered, (1) chemical constitution of abnormal bodies present in the serum; and (2) abnormal conditions of molecular adhesion affecting the serum as a whole, causing the globulins to be easily precipitated, and thereby promoting anticomplementary action. In the last two paragraphs it is assumed that the explanation of the relation of the syphilitic serum to complement is based on (1). One might set up an alternative explanation based on (2), and it might be impossible to decide dogmatically which is right. In this case, an argument in favour of (1) is the probability that, if Hesse's test were based on the physical properties of the syphilitic serum as a whole, it might be arranged to give stronger reactions which would make it independent of two very unsatisfactory conditions,

absolute minimum of complement and amboceptor. But this he does not seem to have found possible. At all events there is room for the alternative postulate that abnormal substances in syphilitic serum are attracted to complement by a very weak chemical affinity which cannot be made stronger by changes in quantity or concentration of the two reagents.

Comparison of Anticomplementary Action in Syphilitic and Normal Sera.

Much experimental research has been based on the idea that the substance which gives a positive Wassermann reaction is present, in a latent form, in normal serum. The elaborate analytical methods of comparison which have developed out of this conception are dealt with in a later section (pp. 154–67).

I begin with a simpler way of expressing this hypothesis. It has been suggested that normal serum contains an antibody which is the same as the syphilitic antibody, the only differences being that it is less in amount, and, like normal agglutinins as compared with acquired agglutinins, less resistant to heat

Friedericke Krauss (1915)* started from the fact, demonstrated by Tojosumi, Spät, and Nakano, that the complement-binding material of syphilitic sera was fixed by certain organ emulsions. An emulsion of liver cells, for example, deprived the sera of their power of binding complement, whilst the cells used for treatment acquired the capacity of fixing complement. Moreover, it had been shown that normal sera could be made to give a positive Wassermann reaction by treatment with various reagents, such as chloroform, though they lost this property on heating, whereas the property was thermostable in syphilitic sera.

Krauss proceeded to compare the action of organ emulsions on normal sera, normal sera treated with chloroform, and syphilitic sera. She used 2 c.c. of each, and digested it with 2 gms. of emulsion of guinea-pig's liver at room temperature for one hour, using as a control 2 gms. of liver digested with 2 c.c. of normal saline. The tubes were then centrifuged, and deposits and liquids were tested separately for the Wassermann reaction. All the liquids were negative. The deposits behaved as follows:—

Amount of Liver Emulsion.	Treated with			
	Normal Serum.	Chloroformed Normal Serum.	Syphilitic Serum.	Normal Saline.
0·5 c.c.	0	0	0	+ + + +
0·25 c.c.	0	0	0	+ + + +
0·1 c.c.	+ + +	+ + +	0	+ + + +
0·05 c.c.	+ + +	+ + +	0	+ + + +

0 = strong inhibition; + + + = well-marked lysis; + + + + = complete lysis.

* *Biochem. Zeitschr.*, LXVIII., p. 48.

The above is an example of the result which she usually obtained. In an exceptional case she found a serum which refused to give a positive Wassermann reaction after chloroforming and also gave a negative result after treatment with liver emulsion.

Her conclusions were:—(1) The "antibody" giving a positive Wassermann reaction was present in normal serum and was not newly-formed by chloroforming or other treatment; (2) the antibody in syphilitic sera differed in being greater in amount and in being thermostable; (3) these differences were similar to the differences between normal and immune agglutinins.

It is not clear that the reactions obtained with normal active serum, whether chloroformed or not, are the same as the reaction with syphilitic serum. The explanation may be that in the former cases the liver emulsion either (1) produces and adsorbs from the serum an anticomplementary substance or (2) simply adsorbs an anticomplementary substance which had previously been produced by chloroform; in the latter, it adsorbs syphilitic antibody. When the Wassermann test is employed, complement is destroyed in the former case by the artificial anticomplementary substance, in the latter case by the combination of antigen and antibody.

Unfortunately, the problem is probably much more complicated than Krauss appears to think. Like many other investigators, she seems to take it for granted that the substance in a normal serum artificially made positive is really the same as Wassermann substance. But serious doubts have been raised on this point. These doubts have arisen partly out of inquiries into laboratory technique, and partly as the outcome of direct comparative experiment. The following are examples illustrative of this sceptical attitude towards "artificial positives."

R. Ottenberg (1916)* made some experiments on the optimum dose of antigen for the Wassermann test, using an alcoholic extract of ox heart. Taking graduated doses of antigen, he determined the "correct" amount of complement for each, *i.e.*, "the least dose of complement that gave complete "laking or a minute fraction above this." This estimation was done both with antigen alone and with antigen in the presence of normal serum. The sheep's corpuscles (one dose) were sensitised with two units of amboceptor. In the test proper, each dose of antigen, with its "correct" amount of complement, was tested against progressive dilutions of a known positive serum.

He found that the largest dose of antigen he used, 3 c.c. of 1 : 10 (total volume, 5 c.c.), would not so readily detect a weak positive as 2 c.c., and this, again, did not give such good fixation with weak sera as 1 c.c. The last was the optimum, as smaller amounts were less favourable. "The dose of antigen "which gives fixation with the highest dilution of positive

* *Journ. of Immunology*, II., p. 47.

" serum is the optimal dose, and this is not by any means " usually the largest dose tested." He thought the reason was that, " when the dose of antigen is increased beyond a certain " point, the amount of complement which has to be added to " overcome the anticomplementary effect is too great to be fixed " by certain grades of positive serum."

I have quoted this is an introduction to the next abstract, in which the author claims that application of the principle of optimum dosage of antigen brings out differences between true and artificial positives.

Rudolf Müller (1916),* from an experience of 150,000 Wassermann tests in which the alcoholic heart extract recommended by Landsteiner, Müller, and Pötzl was employed, was much impressed by the fact that certain dilutions of this extract gave stronger positives with syphilitic sera than were obtained with more concentrated antigen, though the latter obviously contained more lipoid. The success of the antigen, therefore, appeared to depend on its being present in the optimum physical condition.

This observation led to the adoption of a special routine control in addition to the usual controls. For each test concentrated antigen (C) was used, and also that dilution of antigen (N) which was found empirically to give the optimum reaction with a syphilitic serum. It was found that in doubtful cases which were not really syphilitic the N antigen did not give the stronger reaction, but was usually much weaker than the C antigen. This control he came to regard as indispensable. In almost all cases it decided between the syphilitic or non-syphilitic nature of incomplete reactions, and it also eliminated error due to exceptionally sensitive complements ; such complements might give a false positive, but then the N reaction would be weaker than the C reaction.

Referring to the work of Hirschfeld and Klinger, who converted normal sera into positives by shaking in saline, the author stated that he and Buchwald had shown, by using the above control, that such artificially treated sera failed to give the true positive reaction.

Ernst Nathan (1918)† digested 50 normal active sera with distilled water for 24 hours (0·5 c.c. of serum to 4·1 c.c. of water) in the incubator and in the ice-chest. He then applied the Wassermann test, with the following results :—

—	Negative.	Weakly Positive.	Positive.	Auto-inhibitory.
Treated in incubator - -	41	1	1	7
„ „ ice-chest - -	15	5	6	24

* *Wien. med. Wochenschr.*, p. 1075.

† *Zeitschr. f. Immunitätsforschung*, Orig. XXVII., p. 219.

On varying the concentration of serum he found that in the cold almost every normal serum could be made positive or autoinhibitory at some particular degree of dilution, which differed for different sera. If the optimum dilution was not reached or was over-passed, the modification was not brought about.

He also investigated the action of bacteria on normal sera, using a 24 hours agar slope of *prodigiosus* emulsified in 2 c.c. of saline. The bacterial emulsion was rubbed up and then shaken for several hours in a shaking machine until a completely uniform suspension was obtained; after this it was heated for a short time on the water-bath at 100° C. When the sera were treated at 37° C. he found, with his first strain of *prodigiosus*, that a certain number of them gave a more or less definitively positive Wassermann reaction or became autoinhibitory, but the greater number were unaffected. With another strain of *prodigiosus* the number of positives became considerably greater. When his sera were treated in the ice-chest, they gave a positive reaction much more frequently and more regularly.

Comparing serum concentrations of $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{10}$, and $\frac{1}{20}$, he found that the more dilute the serum the greater was the capacity of the bacterial suspension to make it positive or autoinhibitory.

He selected inulin for the purpose of comparing the action of the same substance in suspension and in solution, using Kahlbaum's inulin, which is insoluble in cold water, but goes into a clear solution on warming at 70° C. for a few minutes. The suspension made normal sera positive, but the solution had no effect.

Sera were not made positive if they were inactivated (at 45°—55° C.) before treatment; but there were exceptions to this rule in the case of treatment with bacteria.

Sera artificially made positive lost this property on heating at 45°—55° C., but here, again, this was not the case with many of the sera treated with bacteria, the difference being due, in the author's opinion, to the influence of bacterial lipoids.

In many cases simple storage had the effect of making artificial positives weaker or turning them into negatives.

Nathan's general views were that the artificial positives were produced by changes in the globulins, and that these changes were not characteristic of syphilis, but were such as occurred, or might occur, in leprosy, tuberculosis, pregnancy, and many other conditions. He, therefore, did not agree with Hirschfeld and Klinger that these artificial changes resembled the changes which were produced under the influence of syphilis. In the abnormality peculiar to syphilitic serum thermostability was as essential characteristic.

As regards the special capacity of bacteria to make heated sera positive and to create artificial positives which were not

destroyed by heat, he considered that bacteria did not only exert a physical influence, like distilled water or a suspension of inulin, but also produced an effect attributable to the substances in the bacterial protoplasma, particularly the lipoids, which was distinct from the property of altering the globulins, and led to a thermostable positive reaction. So, in syphilitic serum, the characteristic changes were due to two factors, (1) an alteration in the physical condition of the globulins, and (2) alteration in the chemical composition of the lipoids in the blood serum, the thermostability of (1) being attributable to (2).

Nathan's main practical conclusion was that in the Wassermann reaction inactivated sera must always be used.

Thermostability is regarded by Nathan as an essential distinction between syphilitic and artificial positives.

Comments.

The question of the resemblances and differences between "artificial positives" and syphilitic sera is worth considering, because, if the resemblances prove to be more important than the differences, they have a bearing on the principles of the the W.R., particularly as to the colloidal nature of the test.

As everyone would like to base the W.R. on clear and simple principles, there is a natural tendency to give favourable consideration to a purely physical explanation, one form of which may be set out briefly in the following paragraphs:—

(1) Artificial positives are created without putting anything new into the serum. They are created by agencies which change the colloidal condition of the serum, thereby making it capable of giving a + W.R. These agencies are of a physical rather than a chemical nature, and work better when the chemical activities of the serum are retarded, or put in abeyance, by dilution and exposure to a low temperature.

(2) The properties of syphilitic sera are essentially the same as those of artificial positives. Syphilitic sera are more thermostable and usually give a stronger W.R., but these are only differences in degree, and are probably due to the fact that the causes which have produced the changed colloidal condition in the syphilitic serum have acted slowly and for a prolonged period. Syphilitic sera are not completely thermostable at 56° C, and artificial positives sometimes survive exposure to this temperature.

(3) As the W.R. depends on the colloidal condition of the serum, which is the same in the syphilitic serum and in the artificial positive, the question as to the particular agency which has produced this condition is of no great moment. Experimentally it has been found that a large variety of agencies, including tissue emulsions and bacteria, will turn a normal serum into a positive; so, with the syphilitic serum, the presence, in the circulation, of abnormal tissue disintegration products will be quite enough to account for the change.

(4) As a positive W.R. is due to the colloidal condition of the serum as a whole and not to the presence of a mysterious "Wassermann substance," the desideratum is to simplify the test for syphilis by making it a direct test for a particular colloidal condition.

(5) This suggests the desirability of perfecting the precipitation tests. The forms of this test which are at present in vogue are not delicate enough to be diagnostic, and so resort has to be made to complement fixation as a more delicate means of detecting the formation of a precipitate. If the precipitation test were perfected, complement would be discarded.

Against the view which I have just set out the following objections may be raised :—

(1) It may be conceded that the behaviour of "artificial positives" largely depends on their changed colloidal condition, but this conception should be expanded a little. As a rule these "positives" are only produced when the serum is treated in the active state. Hence it is probable that, underlying different methods for producing this modification in active sera, the same principle is at work, viz., a disruption and rearrangement of the atom groupings, molecules, and molecular groupings on which the activity of the serum depends. In consequence the treated serum, though different from normal complement, contains elements which, being derived or disrupted from the active molecules of complement, possess an affinity (physical or chemical or both) for normal complement and tend to interact with the latter when it is added. This tendency is enhanced by the colloidal suspension used as antigen in the W.R., which attracts the particles of modified serum to its surfaces and so greatly increases the areas of contact between modified serum and complement.

(2) Artificial "positives" differ from syphilitic sera in several important respects. (*a*) The former are very frequently autoinhibitory towards complement in greater or less degree, and the addition of antigen seems to enhance this tendency rather than to create a new anticomplementary compound ; but with syphilitic sera it is customary to find that, when suitable doses are employed, neither serum nor antigen is anticomplementary alone, or only slightly so, but the union of the two forms a complex which is strongly anticomplementary. (*b*) Artificial "positives" appear to differ from syphilitic sera in the distinction produced between optimum dosage and maximum dosage of antigen. (*c*) The difference in heat resistance between the two kinds of sera is much too prominent to be explained away as a minor and inconstant attribute. In view of these differences, it might be argued that, if syphilitic serum is really identical with artificially positive serum in its colloidal condition, this condition does not suffice to explain the W.R.

(3) Hence one cannot reject the view that an essential part in the W.R. is played by "Wassermann substance," meaning thereby some abnormal constituents of the serum as distinct from the physical condition of the serum as a whole.

(4) It is therefore unjustifiable to assume that the W.R. might be reduced to a direct test for a particular colloidal condition.

(5) Syphilitic sera are more readily precipitated than normal sera, but so are sundry other abnormal sera which are not syphilitic. Making the test highly sensitive for the detection of a precipitate is therefore not enough for diagnostic purposes, even if it be conceded that the formation of a precipitate and its deposition on the particles of extract are essential to the W.R. The further requirement is to prove that the precipitate comes from a syphilitic serum. The test must be framed so as to detect not merely a precipitate but a special precipitate, that attributable to a syphilitic serum. But complement alone cannot exercise this selective action; it must be assisted by antigen. The antigen, however, is not specific. One way of meeting the difficulty is to say that there is a selective interaction between "antigen" and "Wassermann "substance" which is of the same nature as the interaction between true antigen and antibody. One would prefer a more precise explanation, but it does not appear to have been found in purely physical theories of the W.R.

Comparison of Split Products of Syphilitic and Normal Sera.

It has been thought that more precise information about the peculiar properties of syphilitic sera might be obtained by chemical analysis, followed by comparison of the components thus obtained with the components obtained from normal serum by similar treatment.

Friedemann's Work.

Ulrich Friedemann (1910), in an experimental investigation of the theory of the Wassermann reaction,* commenced his inquiry by submitting normal sera from various species of animals to the Wassermann test, the lecithin precipitation reaction, the sodium glycocholate precipitation reaction, and the Clausner test (precipitation with distilled water). For the first three tests the serum was heated at 56° C. for half an hour; for the last, fresh active serum was used. Whilst noting that in some of the tests differences occurred between individuals

* *Zeitschr. f. Hygiene*, LXVII., p. 279.

of the same species, he was able to classify his general results as follows:—

——	Ox.	Sheep.	Goat.	Horse.	Dog.	Goose.	Rabbit.	Guinea-pig.	Hog.
Wassermann	0	+	++	0	++ 0	0	+++	0	0
Lecithin -	+++	+	++	0	+	+	++	—	+
Sodium glycocholate.	++	++	+	0	0	++	0	+++	0
Clausner -	++	+	—	++	+−++	—	0	+−++	0?

+++ = very strong reaction.
++ = strong reaction.
+ = weak reaction.

The frequency with which positive results were obtained by one or more of the tests employed, and the lack of correspondence between the results of these tests, might suggest that normal sera commonly contained one or more of four distinct and independent substances, irregularly distributed in different species and each responsive to the particular reagent which gave a positive reaction. This assumption, Friedemann considered, was highly improbable; he preferred, as an alternative explanation, the hypothesis that the substances requisite to give positive reactions with each test were present in all normal serum but might be inhibited by antagonistic influences. He therefore proceeded to see if he could find support for this latter view by demonstrating in normal serum (1) a Wassermann reacting body and (2) a substance antagonistic to this reaction.

As immunological reactions were usually associated with the globulin fraction of the serum, he treated the fresh serum by half-saturation with ammonium sulphate, and examined deposit and filtrate, after dialysis, for evidence of properties corresponding to (1) and (2).

On testing the globulins of normal human sera he obtained the following results:—

(1) Eight cases gave positive Wassermann reactions, but in the absence of extract the globulins, though retarding hæmolysis, were not actually inhibitory.

(2) In nine cases the globulins were inhibitory both with and without extract, but the reaction was definitely strengthened by the presence of extract.

(3) Four cases gave equally strong inhibition with and without extract.

(4) Six cases gave no inhibition either with or without extract.

Investigation showed that the above differences could not be accounted for by any difference in technique, *e.g.*, by keeping

some sera longer than others before separating out the globulins. It was also noted that the properties of the serum yielded by the same person were not constant. For example, the globulin-fraction of the author's serum was strongly inhibitory on one occasion, but serum taken some months later gave a completely negative result.

The results grouped (1)—(4) were obtained with half-saturation by ammonium sulphate. Friedemann thought their irregularity might be accounted for by the presence, in the globulin fraction, of varying amounts of substance antagonistic to the anti-complementary action of globulin. To test this hypothesis he did paralled experiments with the globulins extracted from the same sera by $\frac{1}{2}$ and by $\frac{1}{3}$ saturation. He gives three examples of these :—

	With Extract.		Without Extract.	
	$\frac{1}{2}$ Sat.	$\frac{1}{3}$ Sat.	$\frac{1}{2}$ Sat.	$\frac{1}{3}$ Sat.
First Serum -	strong +	stronger +	—	+
Second Serum -	very slight +	good +	—	+
Third Serum -	strong +	stronger +	+	stronger +

It appeared, therefore, that in the globulin fraction obtained by $\frac{1}{2}$ saturation some antagonistic substances were present but were not forthcoming in the euglobulin fraction obtained by $\frac{1}{3}$ saturation.

In another experiment he compared the effects of $\frac{1}{2}$ and $\frac{2}{3}$ saturation. The globulin obtained with the former was anti-complementary, both with and without extract, but that carried down by $\frac{2}{3}$ saturation was less anticomplementary with extract and not anticomplementary at all *per se*.

Friedemann regarded the above experiments as supporting the view that the essential anticomplementary action resided in the euglobulin fraction, that the function of the extract was merely to intensify this, and that the irregular behaviour of different globulin fractions was due to the presence of different amounts of antagonistic substance.

His observations on the anticomplementary action of globulin could, he considered, be brought into line with what occurred in other immunological reactions. (1) In experiments which demonstrated the interaction of specific antigen and antibody by means of the complement-fixation test, there was first a precipitation, due to the interaction of the two specific substances and consisting mainly of euglobulin brought down from the immune serum. Then, according to Friedemann's hypothesis, it was the euglobulin, thus set free, which brought about the fixation of complement. (2) Complement disappeared on keeping. This might be because the inter-relationship between the constituents of the serum underwent some change

during storage, and thereby the euglobulin was set free to exercise its anticomplementary action. (3) Pfeiffer and Friedberger found certain "antagonistic substances" in normal sera which were brought into evidence by removal of the normal amboceptors contained in the sera: thus, on treating a normal serum with cholera culture, it acquired the property of inhibiting lysis in a bacteriolytic experiment with cholera vibrios. This again might be explained on the hypothesis that removal from the serum of its normal amboceptors brought into free action the anticomplementary property of the globulins.

In support of his argument that the above immunological processes were all dependent on the same principle, the anticomplementary action of globulin, Friedemann called attention to certain respects in which these processes were analogous. He exposed complement to the action of euglobulin in the presence of 4 per cent. saline; after half an hour the strength of saline was reduced to normal by the addition of distilled water, and sensitised corpuscles were added. In a parallel experiment the euglobulin acted on the complement in the presence of normal saline. It was found that the anticomplementary action of the euglobulin, which was well marked in the control, was inhibited by the concentrated saline. Similarly, increase in the concentration of saline interfered with (1) the fixation of complement reaction for the demonstration of specific antigen and antibody and greatly retarded (2) the spontaneous disappearance of complement from serum on keeping. Again, Friedemann found that when the globulin from a serum, obtained by half saturation, was not in itself anticomplementary, it was made anticomplementary by absorption with sensitised corpuscles, *i.e.*, owing to the removal of normal amboceptor or of mid-piece; this effect, he considered, was perhaps analogous to the demonstration of (3) the "antagonistic substances" of Pfeiffer and Friedberger.

Friedemann found that the globulins from syphilitic sera, obtained by half saturation, gave positive reactions with extract but consistently failed to fix complement when used without extract. This was different from his experience with normal sera, the globulins of which, in 9 cases out of 17, fixed complement without the assistance of extract As he had found evidence that, when normal globulins were negative without extract, this was due to the restraining action of substances (perhaps normal amboceptor or mid-piece) carried down with the euglobulins, he thought a similar explanation might be found for the behaviour of syphilitic globulins. This opinion he confirmed by separating the euglobulin fraction of syphilitic sera and showing that it was definitely anticomplementary without the assistance of extract. In syphilis the "globulins" were said to be increased, as shown by precipitation in the ordinary way; this apparent increase might be due to the carrying down of material which, with normal sera, commonly remained behind in the albumin fraction, and this inclusion of material antagonistic to the action

of the globulins might explain why syphilitic "globulins" differed from the normal in not being auto-inhibitory.

Friedemann found that syphilitic globulins differed from the normal in their resistance to heat. In the case of the syphilitic, heating of the serum at 56° C. for half an hour had little or no effect on their anticomplementary action with extract; but normal sera, which yielded an active globulin fraction both with and without extract, lost this property when similarly exposed to heat. This difference, he observed, was further complicated by the fact that the euglobulin of syphilitic serum, which was anticomplementary both with and without extract, was thermolabile, like the euglobulin of normal serum. Hence he considered it necessary to raise the question whether the antibody concerned in the Wassermann reaction had really anything to do with the globulin reactions of normal serum. It might conceivably be the case that, whilst the euglobulins of both normal and syphilitic sera were anticomplementary, there co-existed in the latter sera a definitely thermostable antibody. But, in Friedemann's opinion, when dealing with substances present in a complex medium, resistance to heat was not always a safe criterion for differentiation, as the property of heat-resistance might be profoundly affected by the medium. He had shown that a normal serum which, when active, yielded anticomplementary globulins, lost this property after the serum was heated to 56° C.; but the same globulins, extracted from the active serum, remained anticomplementary after heating to 56° C., though to a somewhat less degree. The explanation probably was that heating of normal serum "increased" the globulin fraction, *i.e.*, added substances, antagonistic to its anticomplementary action, which would otherwise remain in the filtrate. The difference in heat-resistance, therefore, between the globulins present in normal and syphilitic sera, though attributable to changes in the latter sera, did not necessarily postulate intrinsic differences between the action of normal and syphilitic globulins. A positive syphilitic serum would give a negative Wassermann reaction when tested in hypertonic salt solution, which showed, in Friedemann's opinion, a strong feature of resemblance (susceptibility to hypertonic saline) between normal and syphilitic globulins. The Wassermann reaction, in his view, was not a special case of specific antibody-antigen reaction, but was a manifestation of a more general principle, the anticomplementary action of globulin, which underlay but was not confined to demonstrations of specificity by the fixation of complement.

Though some normal sera were anticomplementary in the active state owing, according to Friedemann, to the preponderating influence of their globulins, this was the exception; the majority were not anticomplementary, but might even promote hæmolysis. This interference with the action of the globulins Friedemann had shown to be due to the influence of the

albumin fraction. When he employed separation by half-saturation, addition of albumin to globulin usually annulled the anticomplementary action of the latter. In instances where this was not the case, he thought part of the albumin fraction might have been carried down with the globulin, and he found, by third-saturation, that the well-marked anticomplementary effect of the euglobulin was, in fact, annulled by the addition of the pseudo-albumin, or at least greatly reduced.

Heating of the albumins for half an hour at 56° C. completely destroyed their "antiglobulin" property. Friedemann did not anticipate that this property would be found to have anything to do with either normal amboceptor or mid-piece of complement, because these substances were brought down with the globulin fraction; and he found experimentally that the action of the pseudo-albumin fraction was not interfered with by treatment with washed corpuscles or with sensitised corpuscles.

Syphilitic albumins and pesudo-albumins were found to be quite as potent as those from normal sera in their inhibitory effect upon normal globulins, though they did not affect syphilitic globulins, whether the latter were heated or unheated. The peculiarity of syphilitic serum appeared, therefore, to reside in the globulin and not in the albumin fraction.

As a result of further research on the inter-relationship between globulins and albumins, Friedemann was led to attach considerable importance to what he termed the "dilution phenomenon." Working with normal sera he found that, though the pseudo-albumin fraction antagonised the globulin fraction when the two were mixed in concentrated solution and then diluted, this result was not produced when the two were diluted separately and then mixed. Under the latter conditions, therefore, the normal globulins behaved like syphilitic globulins. He demonstrated this "dilution phenomenon" in experiments both with and without extract, and was equally successful when he employed dialysis with very slightly acidified distilled water for precipitating the globulin fraction, instead of partial saturation with ammonium sulphate.

Friedemann's "dilution phenomenon" led him to postulate the intervention of a third substance, the soaps present in serum, in the interactions between globulins and albumins. According to this view, the soaps attached to the globulins enabled the latter to exert an anticomplementary action, which was reinforced when the globulins could borrow similar material from the "antigen." In this connection Friedemann thought it of interest to note that reinforcement of serological properties by organ extracts had also been observed in bactericidal reactions and in the coagulation of blood. But albumins also could unite with soaps, and so could remove this essential adjuvant from the globulins. In dilute solution the soaps underwent hydrolytic dissociation, and, consequently, a

change was produced in the relations of these bodies to the albumins and globulins.

Thus the negative reaction of normal sera was to be regarded as correlated with the conditions of concentration under which the individual elements necessary for blood formation entered into the blood plasma. A disturbance of these conditions, it was postulated, occurred in syphilis, leprosy, malaria, and certain other conditions; and under such circumstances the blood gave a positive Wassermann reaction. In syphilis, however, Friedemann thought that this simple explanation might not be sufficient; a disturbance in the normal antagonism between globulin and albumin might be brought about in another way, viz., by the entrance of new substances into the serum.

The nature of complement Friedemann regarded as still obscure. As mid-piece was brought down with the globulin fraction, whilst end-piece remained with the albumin fraction, and as mid-piece might become so modified that, on union with end-piece, complementary action was no longer effective, Friedemann devised experiments to ascertain whether the presence of mid-piece in the globulin fraction was in any way associated with the anticomplementary action of globulin, and whether presence of end-piece together with albumin influenced the antiglobulin effect of the latter. But he found no evidence that either mid-piece or end-piece acted in the way suggested. His general conclusion was that complement action was probably not due to a particular and separate substance but would be found to be dependent on the interplay of many factors, which might promote or inhibit this action, just as the phenomena of blood-coagulation had recently been found to involve many factors and to be much more complicated than a simple interaction between two substances termed "fibrinogen" and "fibrin-ferment."

Comments on Friedemann's Work.

As regards Friedemann's main idea, it is generally accepted that metabolism depends upon an extremely intricate and delicately balanced mechanism and that this balance is upset in disease, with the consequence that certain forces, being no longer properly balanced, become "antagonistic." But Friedemann's method of elaborating this conception gives rise to some difficulties.

Perhaps something is to be learnt from his experiments on the sera of various species of normal animals. Different species respond differently to the Wassermann test; moreover, the response of each species to this test does not coincide with its response to precipitation tests for syphilis.

This latter observation evidently suggests that the principles underlying the W.R. are not the same as those on which precipitation tests are based.

The results of the W.R. are difficult to explain on a "balanced antagonism" theory, because all the animals are normal and therefore the sera of one species ought to be as well "balanced" as the sera of another. The observed differences in the response to the W.R. seem to me to involve two consequences; (1) they weaken the suggestion that the positive reactions in normal animals are due to a "disturbance of balance," and hence (2) they lead one to think that, though a syphilitic human serum *may* be "unbalanced" as compared with a normal human serum, this difference does not explain why the former gives a positive and the latter a negative W.R.

In view of this difficulty one must attach more importance to the alternative explanation which Friedemann appears inclined to dismiss, namely, the view that substances giving a + W.R. are present in the sera of some animals and absent in others. This is not an unreasonable assumption; it is analogous to the recognised occurrence, and irregularity of distribution, of normal immune bodies in different species; and in some respects, though perhaps not in all, "Wassermann substance" does behave like an antibody. On this explanation, one has only to postulate that there are reacting substances (not necessarily identical) which respond to the W.R., and that these are natural to some animals but are only found in man as an acquirement due to syphilitic infection.

When normal human sera are artificially split up by a precipitation method, Friedemann has found that the globulins, and more particularly the euglobulins, are anticomplementary. This means that the chemical structure and physical condition of these globulins are of such a nature as to cause loss of activity in the labile elements of fresh serum which come into contact with them. He also finds that the anticomplementary property may be increased by the aid of tissue extracts like those employed in the W.R. The explanation is, no doubt, purely physical. The globulin is attracted to the surfaces of the fine particles of extract; the areas of contact between globulin and active serum are consequently increased, and increased anticomplementary action is the result. These are interesting laboratory data, but it is not obvious that they throw any light on the physiology of either serum or plasma.

Splitting normal serum into "globulins" and "albumins" does not resolve it into its natural constituents, and it may be doubted whether the test-tube reactions of the globulin or the albumin fraction, or of a mixture of the two, help to explain the mechanism of natural processes. It may be conceded, perhaps, that, if one splits a serum and then puts the pieces together again, without altering them by heat or dilution, something more or less like the original serum may be restored. Hence, if the original serum was not anticomplementary, it would not be surprising to find that the restored serum was not anticom-

plementary. But such a discovery is in no way a demonstration that there exists in nature a "balanced antagonism" between globulins and albumins.

In precipitating with ammonium sulphate, more of the contents of the serum comes down with $\frac{1}{2}$ than with $\frac{1}{3}$ saturation, and, again, more is obtained with $\frac{2}{3}$ than with $\frac{1}{2}$ saturation. Consequently, when the precipitates are dissolved, (1) the yield with $\frac{2}{3}$ saturation will be most like the original serum, (2) the yield with $\frac{1}{2}$ will be less like it, and (3) the yield with $\frac{1}{3}$ still less. So one may expect to find, if the original serum was not anticomplementary, that (1) is less anticomplementary than (2), and (2) less than (3).

Nor is it clear that Friedemann's analytical method does much to elucidate the nature of syphilitic sera. Whatever may be the reason, it has to be admitted as a fact that syphilitic sera are more readily precipitated than normal sera. Hence the "globulins" obtained by half saturation are more bulky in the former case than in the latter, *i.e.*, they approximate more to whole serum. Possibly this may suffice to explain why, in Friedemann's experience, the globulins of syphilitic serum are not in themselves anticomplementary, though the globulins of normal serum frequently are. And the reason why syphilitic euglobulins resemble normal euglobulins in being anticomplementary may simply be that $\frac{1}{3}$ saturation brings down, in each case, a less bulky precipitate which is less like whole serum, and is more of an artefact with anticomplementary properties.

But syphilitic "globulins" are highly anticomplementary when assisted by extract. The natural explanation is that this effect is produced not by the globulins, as such, but by the "Wassermann substance" carried down with them. The mechanism would thus be essentially different from the merely physical influence of extract in enhancing the anticomplementary properties of globulins from normal serum, though the latter influence may also play a part in the syphilitic reaction, *i.e.*, anticomplementary activity of some of the constituents of syphilitic serum may be partly due to their adsorption on the surfaces of the particles of extract.

It is also quite possible that the presence of this "Wassermann substance" may be the influence which makes syphilitic globulins come down more readily than normal globulins.

Heating at 56° C. brings out a difference between syphilitic and normal sera. It may be conceded to Friedemann that this fact alone does not prove that syphilitic sera contain a third substance which is thermostable, is independent of the globulins, and possesses the characters (more or less) of an antibody. One must recognise, as he points out, that, when substances are in a complex medium, heat-resistance is not always a safe criterion for differentiation, as this property may be profoundly affected by the medium. At the same time, when this observed difference is taken not alone but in conjunction with other

evidence pointing to the existence in syphilitic serum of a substance not present in normal serum, it may be regarded as corroborative of that evidence. Friedemann does not like this view, because he is anxious to prove that the W.R. is a demonstration of the anticomplementary action of globulin, and that it is not a specific antibody-antigen reaction. But he cannot altogether escape from it. For his own explanation he has to call in the aid of a third factor, the influence of varying degrees of concentration upon the part played by the soaps present in the serum. And he concedes that this supplementary postulate may not be enough, as is shown by his frank admission that in syphilis the disturbance in the "normal antagonism" between globulin and albumin may be due to the entrance of new substances into the serum.

Observations of Schmidt and Hecht.

P. Schmidt (1911), starting from the observations of Porges and Friedemann on the dependence of the Wassermann reaction upon the globulin fraction, investigated the physico-chemical aspects of this relationship.*

In preliminary observations on sedimentation, he found, in agreement with Jacobsthal and others, that, if a mixture of syphilitic serum and extract was incubated at 37° C. and then centrifuged or allowed to stand for a long time, a sediment formed more rapidly and more definitely than in the control tubes containing normal serum and extract. He obtained the same result whether complement was present or absent. In 20 experiments there was, however, one exception, where a negative serum gave earlier and more definite precipitation than the parallel test with a positive serum.

He then investigated the electric charge of his antigen (Lesser's ether-extracted human heart) and found that the particles of the extract-colloid unmistakably possessed a negative charge. When he added to the extract the albumin fraction of normal or syphilitic serum, the electric charge was unaltered. But addition of the globulin fraction to the extract produced a different result. The mixture rapidly became turbid, with a tendency to precipitation, and had to be shaken at the beginning of the experiment. The space surrounding the electrodes became clearer and the bulk of the mixture collected in the middle of the tube but with a tendency in the direction of the negative pole. It thus appeared that the globulin neutralised the electric charge of the extract and that this change was the condition upon which precipitation depended.

Schmidt proceeded to compare the action of albumin and globulin upon extract in the following way. He found that a mixture composed of 2 c.c. diluted extract + 2 c.c. normal saline + 0·3 c.c. of 0·1 per cent. potash alum solution yielded

* *Zeitschr. f. Hygiene*, LXIX., p. 513.

a precipitate in a few minutes when placed in a water-bath at 37° C. But the addition of 0·5 c.c. of 0·1 per cent. solution of albumin in normal saline either completely inhibited precipitation or retarded it for several hours; globulin solution, however, did not exert this inhibitory action until its concentration was raised to 1 : 80; when it was in lower concentration than this, precipitation occurred promptly. He also noted that precipitation was hastened if the globulin solution showed the slightest opalescence (owing to admixture of larger particles of globulin colloid), whereas a turbid albumin solution (obtained by precipitation with alcohol) had no such effect. On mixing albumin and globulin, but keeping within the protective range of the albumin, he found, in his experiments with alum, that an extremely fine flocculation was gradually produced, but no precipitation or sedementation. No difference in this respect was found between the globulins of normal and syphilitic sera.

In Wassermann tests with albumin and globulin solutions, he again found that a turbid globulin solution was much more potent, in the presence of extract, than one which was perfectly clear. Far example, he obtained clear evidence of inhibition with a 1 : 3000 weakly opalescent dilution of a globulin. With solutions of albumin, even in concentration of 1 : 100, lysis occurred just as with normal sera. When albumin was mixed with either normal or syphilitic globulin, the protective action of the albumin was impaired.

He particularly noted that normal sera to which he had added a trace of turbid globulin colloid gave excellent positive Wassermann reactions, whilst lysis was quickly produced in the controls without extract.

His proposed explanation of the Wassermann reaction was that, when the globulins preponderated, either quantitatively or qualitatively (*i.e.*, in their physico-chemical influence), they neutralised the electric charge of the extract-colloid and so caused the formation of new free surfaces in the form of minute globulin particles and enlarged particles of extract; owing to these changes, which were of a progressive nature, complement adsorption took place on the newly formed surfaces.

Hecht (1915),* in a discussion on the Wassermann reaction and its relation to precipitation tests, recommended his antigen, which he employed in the latter tests, as being also useful for the W.R. It was prepared as follows: He placed in a mortar 0·9 gm. of salt and 20 c.c. of alcoholic heart extract, and evaporated to dryness in the incubator. The salt and yellow deposit were rubbed up and then distilled water was gradually added up to 100 c.c. Even after long standing this emulsion showed no noteworthy turbidity.

For the precipitation test he used 0·2 c.c. of patient's serum, active or inactive, and 1 c.c. of his antigen emulsion in a tube

* *Zeitschr. f. Immunitätsforschung.*, Orig., XXIV., p. 258.

of 5 m.m. diameter. After eight hours a cloudy turbidity appeared in the upper third of the tube with syphilitic but not with normal serum. If, however, too much antigen was used, a precipitate might be formed with normal serum.

On carefully pipetting off the turbid material into 1 c.c. of normal saline, shaking, adding complement, and incubating for an hour, he found that there was no hæmolysis on the addition of sensitised corpuscles. On the other hand, the clear fluid did not give fixation of complement. If, however, antigen was added to it, complement was fixed; therefore the clear fluid contained antibody but no active antigen. Similarly in the W.R., he thought, complement was fixed owing to the formation of a precipitate which was invisible.

He tried different combinations of the three reagents used in the first part of the W.R., mixing two of them together, incubating for an hour, and then adding the third, which had previously been warmed. The tubes were then shaken and, after a fixed interval of 5–15 minutes, sensitised corpuscles were added. He found that complement fixation was strongest in the tube where the two reagents heated together were patient's serum and complement; the reaction was complete in 10 minutes, and was quite as good as when all three reagents were incubated together. From his precipitation experiments he had expected that incubation of patient's serum and antigen would have given the strongest fixation.

He regarded the W.R. as a precipitation reaction between colloids of opposite charge, and considered that P. Schmidt's work was important as showing that the differences between syphilitic and normal globulins were not quantitative (degree of electric charge), but probably dependent on differences in the chemical constitution of the colloids.

On chemico-physical grounds, he regarded complement not as an individual substance but as a function of various substances, *i.e.*, "a chemico-physical function of the serum constituents."

Comments on Schmidt and Hecht.

Schmidt's electro-chemical work amplifies and helps to explain Friedemann's laboratory data, but does not make those data any more satisfactory as an explanation of the W.R. Friedemann's analytical method of splitting sera into globulins and albumins and then examining the fractions was disappointing, because, instead of progressing to something new, it kept bringing one back to the starting-point, which simply was that, for some unknown reason, syphilitic serum yielded a precipitate more readily than normal serum. No advance is made from this starting point by showing that the reactions of Friedemann's split products are associated with differences in electric charge.

Perhaps the work of Schmidt and Hecht is of more importance as representing current views* of physicists on a question which does not necessarily involve acceptance of Friedemann's theories about globulins and antagonistic influences. Is the W.R. due to a special chemico-physical condition of syphilitic serum which enables it to form with antigen a colloidal precipitation compound possessing anticomplementary properties?

On p. 162, after discussing the significance of artificial "positives," I came to the conclusion that something more than abnormal precipitability of the serum is needed to explain the specificity of the W.R. Usually one invokes the aid of "Wassermann substance." Do the physicists gain anything by substituting for this term the phrase "chemico-physical condition of the serum"? If they mean a certain condition, or state, of special constituents of the serum, that seems to be very much the same thing as Wassermann substance. But if they mean a special state of the serum as a whole, it is difficult to explain specificity, without the vague and arbitrary hypothesis that a false positive is really in a different "state" from a true positive.

Hence it would seem difficult to obtain the ideal precipitation test which should be infallible and dispense with the use of complement. Such a test, as the physicists admit, would have to depend on selective action due to the special chemical constitution of colloids present in syphilitic serum. One must concede that a remarkably wide range of selective action may sometimes be attained by very slight physical differences. For example, in recent work on acid agglutination it has been shown that very slight differences in degree of acidity of the medium serve to discriminate between the colloidal condition of certain different species of bacteria. Though it has not been proved that such tests are capable of replacing the ordinary method of diagnosis by agglutination, they are certainly evidence of selective action attained by simple physical means. Similarly, if one could pick out from the serum a particular colloid and identify it as syphilitic colloid, this colloid might be found to differ in its physical reactions from other colloids found in serum. But it has not been found possible to isolate such a colloid, and therefore it is difficult to be convinced that the precipitation test, if brought to perfection, might infallibly distinguish between syphilitic and non-syphilitic abnormalities of the serum.

It is instructive to find that the physicists cannot explain reactions such as the W.R. on the relatively simple principles which obtain with inorganic colloids. They are compelled to fall back on chemical constitution, and this, in the case

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* *See also* the views quoted in the *Medical Supplement* compiled by the Medical Research Committee, Vol. I., 1918, p. 14 and p. 44, and the *Bulletin de l'Institut Pasteur*, Vol. XVII., 1919, pp. 35–40.

of proteins and protein derivatives, is highly complex and obscure.

The physicists and biochemists (*see* Hirschfeld and Klinger, p. 176), also point out that a particular property of fresh serum may be due to its chemico-physical state, and not to a particular substance in the serum. Fresh serum, for example, exhibits a particular property termed "complement"; heating at 56° C. changes the state of the serum, and, in consequence, this property disappears; but these facts do not justify the assumption that "complement" is a special thermolabile substance which might be isolated if the chemists were skilful enough. Their observations are highly important, but one must not allow the term "chemico-physical" to obscure their more definitely chemical aspect. Heating at 56° C. destroys certain chemical affinities which are dependent not so much on fixed, or relatively fixed, chemical constitution as on unstable atom groupings or side-chains loosely attached to the molecules. So in dealing with colloidal reactions, at least when fresh serum is concerned, regard must be paid not only to chemical constitution and colloidal condition, but also to these labile chemical activities.

Putting the above considerations together, I think I may add something to the criticism that Friedemann's analytical method does not promise helpful results. One needs a less artificial method, which will take into combined consideration three general properties of the reagents used in the first part of the W.R., viz., (1) the chemico-physical activity of the labile elements in the fresh serum, (2) the chemical constitution of the reagents, irrespective of these labile processes, and (3) the colloidal reactions of the reagents.

Comparison of the Wassermann Test with a Physiological Reaction (Coagulation Test).

Hirschfield and Klinger, the authors of the "coagulation reaction" (C.R.), have endeavoured to throw light on the peculiarities of syphilitic sera by showing that such sera differ from the normal not only in their response to the W.R., but also in a physiological test for the presence or absence of thrombin formation. On these grounds their work is well worth considering, though it must be admitted that their coagulation test is not likely to replace the W.R. in routine diagnosis.

(1) Theory.

Hirschfield and Klinger (1914–15) published a series of articles on the factors determining the coagulation of blood and their relation to complement fixation, the Wassermann reaction, and the general problems of immunity.*

* *Zeitschr. f. Immunitätsforschung.*, Orig. Vol. 20, p. 51 and p. 81; Vol. 21, p. 40; Vol. 24, p. 199 and p. 235.

The Coagulation Test.

They took as their starting-point the following data concerning the coagulation of blood. This phenomenon depended on two processes, (A) the formation of thrombin (fibrin-ferment) and (B) the precipitation of fibrinogen, *i.e.*, the formation of the clot. (A) Thrombin was formed by the interaction of three factors, viz., (1) cytozyme (also called thrombokinase or thrombozyme), which was a thermostable substance present in cells and blood-platelets, and according to Bordet and Delange was a lipoid belonging to the lecithin group; (2) serozyme (also called thrombogen), which was a thermolabile substance present in serum; and (3) calcium ions. (B) When thrombin was formed it precipitated fibrinogen when the latter was present as oxalate plasma, and therefore not associated with free calcium ions.

In their experiments they used fibrinogen in the form of oxalate plasma, as recommended by Bordet and Delange, and found this preferable to Hammersten's pure fibrinogen solution. As cytozyme they first used watery extract of finely powdered guinea-pig's liver; they also found that Bordet's extract of blood-platelets gave good results. Later, they found that many of the alcoholic organ extracts used for the Wassermann test were serviceable. As serozyme they used serum which was prepared from sheep's oxalate plasma and was free from cytozyme.

Their most important observation was that syphilitic serum differed from normal serum in possessing the property of preventing cytozyme from forming thrombin. This was shown by treating cytozyme with syphilitic serum and then adding serozyme and calcium ions; on testing this mixture with fibrinogen, clotting failed to occur or was markedly retarded. When untreated cytozyme was used, clotting occurred readily, and with cytozyme treated with normal serum clotting again occurred, or was much less retarded than in parallel tests with syphilitic serum, since the inhibitory influence of normal serum on cytozyme was relatively weak, and the difference between the two could be clearly demonstrated by properly adjusted quantitative tests.

Briefly, their technique was to treat 0·1 c.c. of well inactivated serum with 0·1 c.c. of extract, diluted in saline, for one hour. They then ascertained how much cytozyme was still present in the mixture. This was done by adding 0·5 c.c. of diluted serozyme and 1 c.c. of CaNaCl solution; after 15 minutes, oxalate plasma was added and the tubes were watched. The occurrence of clotting proved that cytozyme was still present after treatment with the serum tested; the more rapid the clotting the more abundant the cytozyme. They used graded doses of extract to ensure proper delicacy of the reaction and

recommended (with Merck's extract) 0·1 c.c. of 1 : 40, 1 : 120, and 1 : 360.*

They also investigated the condition of the serum in anaphylactic shock, and found that, when animals (rabbit, guinea-pig and dog) had been actively or passively sensitised towards a particular antigen, on re-injecting the antigen and withdrawing blood a few minutes later, the serum thus obtained exhibited the property of inhibiting cytozyme. The same change was found in the serum of guinea-pigs after injection of anaphylatoxin. Thus in both cases the blood acquired a property which they had hitherto found only in the blood of syphilitics, viz., the property of producing what they termed "a positive " coagulation reaction."†

As regards the properties of cytozyme, they found, like Bordet and Delange, that pure cytozyme obtained from blood-platelets was thermostable. But when cytozyme was present in fresh serum the effect of heating was to cause its disappearance. When organ extracts were used as cytozyme the effect of heating varied. Sometimes they were attenuated, sometimes they were unaffected, and sometimes they were increased in potency. Organ extracts, they thought, contained a mixture of substances which promoted and substances which hindered coagulation; when the balance between these factors was unequal, the effect of heating might be to readjust it, either in the one direction or in the other.

Suspensions of bacteria did not act as cytozyme, or only very feebly; but they became potent when treated with fresh serum containing cytozyme. Similar treatment made suspensions of inorganic powders, such as kaolin, act as cytozyme. Also specific serum precipitates, *e.g.*, the precipitate obtained with bovine serum and rabbit precipitin, acted as cytozyme after treatment with serum containing cytozyme. It was usually found that the cytozyme content of the serum was lowered after treatment. The power of a serum to make bacteria act as cytozyme was impaired or completely inhibited by inactivating the serum or by adding $BaCl_2$ or 2 per cent. NaCl. But neither 2 per cent. NaCl nor $BaCl_2$ interfered with the absorption of cytozyme by kaolin; and, under similar treatment, specific serum precipitates sometimes behaved like kaolin, but sometimes resembled bacteria in failing to absorb cytozyme. They made these experiments because 2 per cent. NaCl and $BaCl_2$ were known to interfere with the thermolabile functions of serum (activity of complement and formation of anaphylatoxin). For example, these salts inhibited fixation of complement by bacteria but not by kaolin.

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* For fuller details of the technique, which was followed by Brandt and Uemura at the same Institute, *see* pp. 180–4.

† To avoid confusion one must be careful to remember that the authors call their reaction "positive" when coagulation fails to occur or is markedly retarded, *i.e.*, when the coagulation reaction is negative.

It was also found that the capacity of organ emulsions to act as cytozyme was enhanced by treatment with serum containing cytozyme.

The power of acting as cytozyme might be acquired by kaolin, bacteria, &c., not only from sera but from other liquids containing cytozyme, such as emulsions of lipoid extracts and extracts of blood-platelets. Such emulsions and extracts acted strongly on kaolin but only weakly on bacteria. It was found, however, that their action on bacteria was intensified by the addition of active serum (free from cytozyme). Thus, they took (1) cytozyme-free oxalate serum and (2) extract of platelets, and showed that (1) failed to make either bacteria or kaolin act as cytozyme, whilst (2) had only a slight influence on bacteria; but a mixture of (1) and (2) rendered the bacteria a potent cytozyme.

Active sera, therefore, possessed three properties, which should be regarded as different functions rather than different substances, viz., (1) complement, (2) serozyme, and (3) the capacity of causing other substances to act as cytozyme. These three were alike in being thermolabile, in being inhibited by hypertonic saline, and in being dependent in some imperfectly understood way upon the interrelationship between the globulins and the albumins of the serum.

Split Products of Normal Sera in Relation to the C.R.

The authors paid special attention to the influence of the globulin and the albumin fractions.

Sera obtained from blood which had been allowed to clot in the ordinary way (subsequently termed "serum") usually contained cytozyme. When such serum was treated with ammonium sulphate or other reagent to separate the globulins from the albumins, the cytozyme came down with the globulins; and when the globulin fraction was redissolved it generally acted more potently as cytozyme than the corresponding quantity of whole serum. In the albumin fraction cytozyme was not usually demonstrable, or was not present in more than small amount.

When sera obtained from oxalate plasma (subsequently termed "oxalate serum") were tested with sheep serozyme, cytozyme was found to be absent or no more than small in amount. And when the globulins of such oxalate sera were similarly tested they were found to be poorer in cytozyme than the globulins obtained from ordinary sera.

Splitting oxalate serum into globulin and albumin fractions was found to have an influence on the formation of thrombin. Such serum was first tested whole, and was found to contain no thrombin; but the globulin obtained from it produced rapid clotting, without the addition of serozyme. The globulin, therefore, contained thrombin. When, however, the globulin, after

its precipitation, was dissolved in an oxalate medium, the result of the test for thrombin was negative. The thrombin, therefore, was not pre-existent in the globulins of the serum but was newly formed out of the precipitated globulin fraction. In the albumin fraction, on the other hand, thrombin was generally found to be absent or only scanty.

When extract of blood-platelets was added to an oxalate serum and the serum was then split into globulin and albumin fractions, it was found that (1) the extract of platelets passed over into the globulin fraction, and (2) the separation into a globulin fraction containing cytozyme and an albumin fraction free from cytozyme was made more complete. In an example given, the albumen fraction of an oxalate serum untreated with platelets contained enough cytozyme to give coagulation in 45 minutes; but the albumin fraction from the serum treated with platelets did not give coagulation until the following day.

The effect of adding albumin to globulin varied in different experiments. Sometimes the albumin restrained the coagulating property of the globulin, *e.g.*, it might delay clotting from $6\frac{1}{2}$ to 120 minutes; and sometimes the albumin accelerated the action of the globulin. It was found that this variation often depended on the quantity of the reagent used for splitting the serum.

Using as their reagent $\frac{n}{300}$ HCl and employing 1·5 c.c. of oxalate serum, they demonstrated the effect of varying the quantity of reagent in a series of experiments, of which the following are examples:—

(1) The globulin brought down with 10 c.c. HCl produced coagulation in 240 minutes; the time was reduced to 90 minutes by the precipitate with 12·5 c.c. and to 30 minutes by the precipitate with 15 c.c. Before treatment with HCl, the oxalate serum contained no thrombin. Parallel experiments with the ordinary serum showed similar but less well-marked variations, according to the quantity of HCl used.

(2) The albumin from an oxalate serum treated with 10 c.c. HCl restrained the action of the globulin; but, when the serum was treated with 15 c.c., the action of the globulin was accelerated by the albumin.

(3) The globulins obtained with 10 and 12·5 c.c. produced coagulation in 22 minutes, and that obtained with 15 c.c. in 30 minutes; the albumin exhibited a retarding action, which increased in proportion to the amount of HCl, the times of coagulation being—with 10 c.c., 120 minutes; with 12·5 c.c., 180 minutes; with 15 c.c., 250 minutes.

(4) 0·1 c.c. of an oxalate serum was tested for serozyme (cytozyme being added) and was found to contain very little, clotting not being obtained until next morning. The albumin obtained with 15 c.c. HCl behaved similarly, but that obtained with 10 c.c. was a potent serozyme, clotting being obtained in 2 minutes; with half the dose, clotting occurred in $4\frac{1}{2}$ minutes, and in 1 hour with a quarter the dose.

The authors regarded the variations obtained by using different quantities of reagent as analogous to the differences found by Friedemann in the effects of $\frac{1}{2}$ and $\frac{1}{3}$ saturation with ammonium sulphate. In each case the physical antagonism between the globulins and albumins appeared to depend on the concentration in which these substances acted on one another,

probably owing to the principle which Friedemann demonstrated by showing the effect of dilution upon anticomplementary properties.

Friedemann had observed that the effect of splitting a normal serum was the removal of a restraining influence exercised by the albumins upon the globulins, the result of this removal being that the separated globulins became anti-complementary, and gave a positive Wassermann reaction. Similarly, it appeared to Hirschfeld and Klinger, disturbance of the normal balance between globulins and albumins might be a factor which determined thrombin formation. This would explain why thrombin might be formed out of the globulin fraction of a serum, though no thrombin was demonstrable in the whole serum, the antagonistic mechanism, which was present in the whole serum and prevented thrombin formation, being removed by splitting the serum.

With regard to the distribution of serozyme in the globulin and albumin fractions, they found that the latter fraction often acted more strongly as serozyme than the whole serum. As globulin alone was capable of producing thrombin, it followed that this fraction of the serum also contained serozyme. They found no evidence that serozyme was separable into an end-piece and a mid-piece; its activity depended on the quantitative relationship between albumin and globulin.

As to the influence of heat on cytozyme, they found that globulins obtained from a serum which had been inactivated (and, consequently, had lost its capacity for acting as cytozyme) were usually weaker in their action than those obtained from active serum. If the globulins obtained from a serum were re-dissolved and then inactivated or boiled, their capacity to act as cytozyme was sometimes weakened and sometimes strengthened. Similarly, with organ extracts, heating sometimes improved their quality as cytozyme and sometimes impaired it.

Methods of making a Normal Serum give a + W.R.

Reverting to the Wassermann test, Hirschfeld and Klinger proceeded to consider various methods of modifying a normal serum so as to make it give a positive reaction to this test.

In their experiments on the modifications produced in serum by shaking, they used normal human serum, usually fresh, or after keeping for 12—24 hours in the cool room. The serum, either neat or diluted 1 : 3, 1 : 5, or 1 : 10, was shaken at room temperature for ½—5 hours. They used flasks of two sizes, 30 c.c. flasks for not more than 5 c.c. of liquid, and 60—65 c.c. flasks for not more than 10—15 c.c. of liquid. After shaking, the serum was submitted to the Wassermann test, for which they used doses of 2 and 1 c.c. of antigen (liver extract), and 0·05 c.c. of complement.

The following is an example of a test with 0·1 c.c. of normal active serum diluted 1 : 10 in normal saline. The table shows the amount of hæmolysis obtained. The control was not shaken, but was allowed to stand for some time in the diluted condition before testing.

——	Serum shaken for 30 minutes.	Control.
2·0 extract	0	Almost complete
1·0 „	Trace	Complete
No „	Complete	„

It will be noted that mere standing in the dilute condition, without shaking, had a slight influence. In some of their experiments this influence was much more marked, though the controls (serum added without dilution), always gave complete hæmolysis.

In another experiment active serum, diluted 1 : 10 in normal saline, was shaken for 90 minutes, and was then divided into three portions. The first was tested without further treatment, and gave complete inhibition of hæmolysis. The second was centrifuged and tested after removal of the globulins precipitated by the shaking; the same result was obtained as with the first portion. The third was inactivated and then tested; the result was complete lysis.

Their general experience was that the property of giving a positive Wassermann reaction was developed most strongly in active normal sera which had been shaken in 1 : 5 and 1 : 10 dilution with normal saline, though a change in this direction was produced when the dilution was only 1 : 2.

When normal serum was inactivated before shaking and then diluted, shaking had no effect, the result of the Wassermann test being complete hæmolysis.

Similarly, shaking of active normal serum in dilutions of hypertonic saline (2 per cent.) failed to produce active Wassermann substances, though the dilutions were made isotonic before applying the Wassermann test.

When shaking was prolonged, *e.g.*, for 4 hours, inactivated serum still remained unaffected, whether diluted or not; and prolonged shaking did not overcome the inhibitory effect of 2 per cent. saline on active serum, though it produced as much turbidity as in the control (active serum diluted with normal saline), and the latter gave a positive Wassermann reaction after shaking. Prolonged shaking of active serum tended to stabilise the Wassermann reacting substance which was produced. They observed, for example, that, with some sera, this substance, when obtained after a short period of shaking, was lost when inactivated; but when inactivated after a long period of shaking it was retained. Shaking of the serum in higher

dilution also promoted the resistance of the Wassermann substance to inactivation by heat. The difference, therefore, between syphilitic sera and normal sera artificially made positive appeared to be merely quantitative and not qualitative.

The effect of diluting active normal sera in distilled water (1 : 10), without the assistance of shaking, was to make them anticomplementary, and capable of giving a positive Wassermann reaction.* But this effect was not obtained with inactivated sera.

The authors' general conclusions from the above experiments were that shaking in normal saline or dilution with distilled water produced in normal active serum a "labilisation" of the protein colloids, leading to a visible turbidity of the globulins; in consequence of this change the sera acquired anticomplementary properties, and gave a positive Wassermann reaction; but this anticomplementary capacity was not attributable to the turbidity produced, because it persisted in the clear liquid obtained after centrifuging. They regarded the change produced by shaking as occurring in the globulin and not in the albumin elements of the serum; and they thought that inactivation or hypertonic saline stabilised the globulins, and so prevented these changes from taking place.

The authors proceeded to observe the effects of treating normal human sera (0·1 c.c.) with suspensions of bacteria, kaolin, and agar for 2—3 hours at 37° C. After careful centrifuging, the clear liquid was submitted to the Wassermann test. Controls were always set up with portions of the serum untreated.

The general result was that agar proved the strongest reagent, kaolin came next, and bacteria last; the influence of agar was not counteracted by subsequent or prior inactivation of the serum. The following details of experimental results may be given :—

(1) Serum active; made positive or anticomplementary by bacteria, kaolin, and agar. Serum inactive; made positive by agar only.

(2) Serum active; kaolin made it weakly positive, but bacteria had very slight effect. Serum inactive; remained unchanged.

(3) Serum active; made positive by both kaolin and bacteria. Serum inactive; unaltered.

(4) and (5) In each case kaolin made the active serum positive, but bacteria failed to do so.

(6) Serum active; made positive or anticomplementary by bacteria, kaolin, and agar. Serum inactive; influence of bacteria and kaolin weaker, but influence of agar rather stronger.

(7) A similar experiment to (6), with the same result.

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* They always used a control tube with nc extract.

(8) Serum active; made positive by bacteria, kaolin, and agar; after treatment with the last reagent, but not with the two first, the liquid was found to be thermostable.

(9) Serum inactive; made positive by bacteria and kaolin as well as by agar (an exceptional result). With the last reagent it was made auto-inhibitory.

In the above experiments the controls (without extract) showed that agar alone produced an auto-inhibitory effect. With reference to the use of agar, they remarked that Bordet had found agar to possess the property of making active serum become turbid. He used 0·5 per cent. agar in normal saline. Hirschfeld and Klinger used agar two or three times more dilute, and found it best to add it in a liquid condition at 40° C. An advantage of agar over kaolin and bacteria was that it was clear, and so any turbidity produced could be readily detected.

In addition to bacteria, kaolin, and agar, they tried the effect of specific precipitates, *e.g.*, the precipitate obtained with bovine serum + rabbit immune serum, a dense suspension of the precipitate being used in place of bacteria, &c., and then removed by the centrifuge. When active serum was used, an anticomplementary effect was produced (slight lysis without extract) and a positive Wassermann reaction (complete inhibition of lysis) was obtained with the aid of extract. With inactive serum, a change in the same direction was obtained, but it was definitely weaker. As with bacteria and kaolin, inactivation of the serum after treatment annulled the effect of treatment, the result of the test being complete hæmolysis. On active serum the precipitate had the same effect in the presence of hypertonic as in that of normal saline.

The C.R. with Sera artificially + to the W.R.

Starting from the facts that (1) syphilitic sera (inactivated) inhibited coagulation, *i.e.*, gave a positive coagulation reaction (C.R.) as well as a positive Wassermann reaction (W.R.), whilst (2) normal human sera (inactivated) were negative to both tests, when carried out with due precautions, they proceeded to consider and compare the effects on the C.R. of those modifications which had been found to make a normal serum give a positive W.R.

Serum shaken.—(1) Active. Whereas the W.R. became positive, the C.R. was usually made more strongly negative than before; the cytozyme content of the serum was increased. Prolonged shaking, which tended to stabilise the Wassermann substance produced, generally caused a gradual diminution in cytozyme content. Shaking did not affect serozyme. (2) Inactive. No effect.

Serum + distilled water.—(1) Active. As above, W.R. made positive; C.R. made more strongly negative, cytozyme action being strengthened. (2) Inactive. No effect.

Serum + bacteria.—(1) Active. Usually made positive to W.R.; C.R. made more strongly negative, though cytozyme action not definitely intensified. (2) Inactive. W.R. usually negative; C.R. as in (1).

In the C.R. tests they used 1 c.c. of serum and 0·2 c.c. of bacterial suspension; it was important to avoid dilution, as this alone might reduce inhibitory action of the serum upon the cytozyme.

Serum + kaolin.—(1) Active. Both W.R. and C.R. made positive. Heating, after treatment, made C.R. negative and usually annulled, or reduced, W.R. (2) Inactive. C.R. negative; W.R. either negative, or, if not, usually reduced.

Serum + agar.—(1) Active. W.R. made positive; C.R. more strongly negative than without treatment. On inactivation after treatment, W.R. still positive and C.R. as before. (2) Inactive. W.R. positive; C.R. negative.

Globulin and albumin fractions of split serum.—(1) Globulin. W.R. positive; C.R. more strongly negative than with the whole serum, the effect of the separated globulin being to intensify the cytozyme of the extract. After inactivation, the action of the globulin was weakened, though remaining less unfavourable to cytozyme than the corresponding quantity of whole serum. (2) Albumin. W.R. negative; C.R. negative, but influence of albumin on cytozyme always unfavourable, though less strongly than the corresponding quantity of whole serum. (3) Globulin + albumin. In both C.R. and W.R. the two fractions had an antagonistic influence on each other (subject to the conditions mentioned on pp. 170–2).

In conjunction with the above observations, they found that, unlike normal globulins, the globulins from syphilitic sera, obtained by either $\frac{1}{2}$ or $\frac{1}{3}$ saturation with ammonium sulphate, inhibited cytozyme, *i.e.*, they gave a positive C.R. The albumins, like those from normal sera, were also unfavourable to cytozyme; and a mixture of albumin and globulin was always more strongly inhibitory than either alone. For the C.R., therefore, there was no antagonism between the globulin and albumin fractions of syphilitic sera.

Conclusions of Hirschfeld and Klinger.

As regards the bearing of their results on general principles of immunity, Hirschfeld and Klinger observed that influences, such as inactivation or hypertonic saline, which increased the stability of the globulins in a serum, had also the effect of inhibiting certain properties of the serum, but this effect did not, of itself, justify the conclusion that those properties of the

serum were to be identified with a thermolabile substance. The more correct inference, they considered, was that the serum could only exercise these properties when its colloids were in a certain degree of "lability," *i.e.*, of capacity for altering their degree of dispersion. Fixation of complement they regarded as due to capacity for labilising the globulins of active serum, though this conception was not intended to exclude the possibility of a simultaneous direct adsorption of complement by suspended colloids. They thought the difference in potency between their different reagents (bacteria, kaolin, &c.) might be due to such differences in primary affinity for complement.

On comparing the value of different extracts for use in the syphilitic C.R. they found that, for a good extract, two characters were requisite, (1) capacity to act as cytozyme and (2) "specific" action on the syphilitic serum. It was not determined whether (1) and (2) depended on the same or on different substances, but it was found that some extracts possessed (1) but not (2), and some (2) but not (1). Thus content of cytozyme did not always correspond with capacity to react with syphilitic sera.

For example, certain acetone extracts, which they found to be free from cytozyme and therefore useless for the C.R., gave a good W.R. Moreover, such acetone extracts did not reinforce the syphilitic C.R. when added to an extract containing cytozyme, *i.e.*, they did not increase the inhibitory effect of the syphilitic serum upon coagulation. In another set of experiments they took cytozyme-free acetone extracts and mixed them with syphilitic sera; then, either immediately or after waiting for a reaction to take place, they added cytozyme in the form of extract of blood-platelets. No change was effected in the activity of the cytozyme.

From the above observations they concluded that in the C.R. they were dealing not with a secondary change in the serum, brought about by the lipoid extract, but with a change produced in the lipoid itself. In the W.R., on the other hand, it was not the character of the extract as cytozyme which was the determining factor but its capacity to produce certain changes in the serum globulins, thereby leading to complement fixation. In both C.R. and W.R. there was a precipitation of globulins in the intermediate stage of the reaction; but whereas in the latter the size of the globulin particles was of minor significance, in the C.R. it appeared to be the determining factor. Upon this factor it depended whether the lipoid particles were surrounded by the adjacent globulin particles (and so became incapacitated as cytozyme), or whether the lipoid particles were adsorbed on the surface of the globulin particles (and so increased their activity as cytozyme).

The principal difference between the C.R. and the W.R. was that in the former the principle serving both as antigen and as indicator (the cytozyme) was contained in one reagent, and, con-

sequently, changes due to the reaction were demonstrated, and quantitatively estimated, directly by the antigen; whereas in the complement fixation test, the indicator, the complement, was only an indirect participant in the reaction. This, they considered, was the reason of the greater delicacy which they claimed for the C.R.

These conclusions may be supplemented by a further article (1914).* In this Hirschfeld and Klinger, after paying a tribute to the work of Bordet and Delange on blood-coagulation, which provided the foundations for their coagulation test for syphilis, admitted that a complete explanation of the reaction was not yet available. It could not be attributed to a direct capacity of syphilitic serum to prevent coagulation, because the serum only manifested its capacity after contact with organ extract. Their suggestion was that there was a colloidal transformation of the serum, a kind of ultra-microscopic precipitation of the globulins, due to contact with the extract. This change in the globulins probably gave rise to an adsorptive process, with consequent diminution in the activity of the cytozyme of the extract. In this connection they made some interesting observations on sera which contained cytozyme and were consequently useless for the test, as coagulation occurred in the controls (without extract). With syphilitic sera rich in cytozyme, the control would give rapid coagulation (2—5 minutes), whereas, in the tubes containing increasing doses of extract, coagulation was delayed, delay being greater with the larger doses of extract. But with normal sera, equally rich in cytozyme, coagulation was more rapid in the tubes containing extract than in the controls.

They compared the C.R. and W.R. in 250 cases, and found that the results were almost completely concordant. In some cases of latent and of treated syphilis, the C.R. was found to be the superior test. Whilst not suggesting that the C.R. would be likely to oust the W.R., they thought it might be a useful control in doubtful or treated cases.

Comments on Hirschfeld and Klinger.

Hirschfeld and Klinger's articles are rather involved, and some points are not expressed very clearly. No doubt this is largely owing to the difficulty of their problems, which, as they admit, have not been fully elucidated. Consequently I have found it difficult to summarise their work, but I have done my best to present their main facts and theories from their own standpoint.

The chief interest in their research lies in their discovery that syphilitic sera influence a physiological reaction, thrombin formation, in a special way, by interfering with the property of cytozyme to act on serozyme. This character of syphilitic sera is not equally well marked in every case, but the authors appear

* *La Semaine Médicale*, 5th August 1914, p. 361.

to have established their contention that, in carefully adjusted experiments, it is a definite feature which distinguishes syphilitic from normal sera. One therefore wishes to consider how far their experiments throw light on the characters which are peculiar to syphilitic sera.

They have devoted a good deal of time to the study of "artificial positives," *i.e.*, normal sera which have been artificially treated in such a way that they tend to become anticomplementary of themselves, and are definitely anticomplementary with the aid of extract. Such sera, they find, differ sharply from syphilitic sera, in that they do not interfere with thrombin formation. This difference provides a reason, which may be added to the reasons already given (pp. 153–4 and 160–3), for regarding "artificial positives" as qualitatively different from syphilitic sera; and it suggests that much caution is necessary when these artefacts are used to explain the characteristics of syphilitic reactions.

Still, something may perhaps be learnt from them. The way in which an "artificial positive" behaves in the W.R. is fairly clear. Something is adsorbed on to the surface of the particles of extract, and by this means its capacity for anticomplementary action is increased. But when the same serum and the same extract, now termed "cytozyme," are used for the C.R., the result is negative. From this it is fairly clear that cytozyme may adsorb serum on to its surfaces without being put out of action.* How, then, does a genuinely syphilitic serum prevent thrombin formation? It has no action on serozyme, according to Hirschfeld and Klinger, so it must act on cytozyme; and, as adsorption does not appear to be a sufficient explanation, it seems necessary to postulate that there is a direct interaction between serum and cytozyme which destroys the property of the latter to act on serozyme. If this is the case, one is led to think that, in the W.R. also, syphilitic serum is not merely adsorbed by syphilitic antigen but effects a structural change in it, because the same extract serves for both "cytozyme" and "antigen."

Hirschfeld and Klinger are strongly influenced by Friedemann's idea of a "balanced antagonism"; they also follow very closely his plan of trying to discover the properties of sera by a highly artificial method of analysis. As I have already discussed Friedemann's work, I have not much comment to add about Hirschfeld and Klinger's elaboration of it. I have quoted their data at considerable length, because they are a very good example of a particular method of analytical research. This method has been very frequently employed by workers on

* Hirschfeld and Klinger say that it depends on the size of the globulin particles whether the cytozyme is surrounded by them (with inhibition of coagulation) or whether the cytozyme surrounds the globulin particles (with increased activity of cytozyme). But this explanation cannot very well hold good for the same extract and the same serum which give a + W.R. and a — C.R.

immunity, and therefore it is of great importance to decide whether it is sound and useful. If it is, well and good; but if it is not, the adoption of it is a serious barrier to progress.

(2) Practical Applications of the Coagulation Test.

Brandt.

Max Brandt (1915), working at Zurich under Hirschfeld and Klinger, the inventors of the coagulation test for syphilis, compared the coagulation reaction (C.R.) and the Wassermann reaction (W.R.) on 500 sera received at the institute during June and July 1914.* The sera, many of which had passed through the post, were treated for $\frac{1}{2}$ hour and then put through the W.R. The next day they were treated for an hour at 58°—60° C. and submitted to the C.R.

Brandt remarked that blood obtained from clot in the usual way contained very little serozyme but generally a good deal of cytozyme; the latter was to a large extent inactivated by heating the serum at 56° C. Like Hirschfield and Klinger, he found that watery emulsions of the ordinary alcoholic extracts used for the W.R. provided a very active cytozyme. If added to normal sera, this property, though slightly weakened, was not depreciated to an important extent; but when the emulsion was digested with syphilitic sera it lost, either completely or to a very large extent, its capacity to act as cytozyme. The extract which he used was Merck's preparation from guinea-pig's heart.

In conducting the C.R., 0·1 c.c. of the serum to be investigated was added to 0·1 c.c. of each dilution of the extract emulsion (*see below*) and the mixture was allowed to stand for one hour at room temperature. Then he added 1 c.c. of salt solution (composed of 100 parts normal saline and 5 parts of 1 per cent. $CaCl_2$) and 0·5 c.c. of diluted sheep-serozyme. Fifteen minutes later, oxalate plasma was added. If thrombin was present, clotting occurred, and the more the thrombin the more quickly was a clot formed. Absence or retardation of coagulation was consequently an indication that the cytozyme of the extract had been wholly or to a large extent inactivated, and was evidence of syphilis. In reading the tubes during the first 15 minutes, several observers were required if there were many sera; after this period, readings every five minutes were enough. In recording results the time required for the formation of a clot was given in minutes; the tubes not clotted in three hours were returned as "fluid" (fl.). Interpretation of the results required consideration of the relative and not merely the absolute time to produce clotting, as the time varied from day to day within certain limits, according to the strength of the serozyme and the coagulability of the plasma.

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* *Deutsch. med. Wochenschr.*, July 25th, 1915, p. 915.

The following is an example of a protocol :—

Clinical Diagnosis.	Progr. Paral.	Optic Neuritis.	Lues III.	Lues I.	Tabes ?	Alcoholic.	+ Control.	− Control.	Extract alone.
Extr. dil. 1 : 40	fl.	1	1	1	2	1	2	1	1
Extr. dil. 1 : 80	fl.	2	2	2	fl.	2	25	1	1
Extr. dil. 1 : 160	fl.	2	25	2	fl.	2	fl.	2	2
									Serozyme alone.
Serum alone	fl.	fl.	fl.	3	fl.	fl.	fl.	fl.	fl.
C.R. result	+	—	weak +	auto-coagu-lating.	+	—	+	—	—
W.R. result	+	—	weak +	weak +	weak +	—	+	—	

In the above experiment coagulation with 1 : 40 extract was too rapid, and the weakly positive sera only manifested their capacity for retarding coagulation with the extract diluted to 1 : 160. In another protocol, given by Brandt, where coagulation was not so rapid, the positive characters of the sera were also demonstrated with the 1 : 80 and 1 : 40 dilutions.

Brandt summarised his results as follows. Both reactions corresponded in 425 cases (94 per cent.). In 20 cases where they failed to correspond the C.R. corresponded with the history and clinical record (tertiary syphilis, tabes, aneurysm, keratitis parenchymatosa, &c.). In three cases with a positive history, one primary and two treated, the W.R. was + but the C.R. was —. In about 100 cases diagnosed clinically as probably not syphilis, there were only two, one of chronic nephritis and one of pernicious anæmia, where the C.R. was +.

Not infrequently (in 49 cases out of 509) Brandt found that the sera produced clotting without extract. This was usually in specimens sent by post, and was due, in Brandt's opinion, not to any inherent fault in the C.R., but to the presence in the sera of so much cytozyme that heating failed to destroy it completely.

He found that the C.R. could be made still more delicate by using smaller doses of extract. At the time of writing he employed in his institute dilutions of 1 : 40, 1 : 120, and 1 : 240. Thereby he obtained more positives than with the W.R., and he usually found that the C.R. corresponded with the history. He thought it would be possible to make the C.R. so delicate, without losing its specificity, as to detect every case of syphilis, notwithstanding the effect of treatment. The C.R., he considered, might owe its greater sensitiveness to its being "a

" direct expression of the changes set up in the lipoids," whereas the W.R. only indicated such changes indirectly by binding complement.

Brandt compared the sera of various species of animals by the W.R., C.R., and Porge's precipitation reaction. The results did not tally, though a good number were + to the C.R. In only one species, bovine, did the W.R. agree with the C.R.

Uemura.

Hisakito Uemura (1917) employed the coagulation test for syphilis at the Zurich Institute, and compared it with the Wassermann reaction.*

For the employment of the former test the following details of technique are given.

Extract emulsion.—He found that almost any alcoholic extract would serve, and he generally used guinea-pig heart or human liver; acetone extracts must be avoided. The concentration and effectiveness of the extract should be such that, when diluted 20 to 40 times and tested as described below, 0·1 c.c. caused the oxalate plasma to coagulate in 1 to 3 minutes. For the purpose of the test it should be diluted 1 : 40, 1 : 80, 1 : 160, and also, perhaps, 1 : 320, and every serum should be tested with each of these dilutions. It was necessary for the extract to be dilute, as in stronger solutions, *e.g.*, 1 : 10, the alcohol present would affect the formation of thrombin.

Serozyme.—From the compressed jugular vein of a sheep or goat the blood was allowed to flow in a jet, through a fairly thick cannula, into a glass vessel containing 1 per cent. sodium oxalate. As this solution was not isotonic for all animals, the author recommended the addition of one-twentieth volume of 10 per cent. saline. The blood was run into the vessel until it reached a mark showing that it then contained one per thousand oxalate. The vessel and oxalate should be previously warmed to 40° C., to avoid risk of laking the blood by condensation water. After the blood had been centrifuged for 10 to 15 minutes in glass vessels rinsed with saline, the plasma was pipetted off into fresh glasses and vigorously centrifuged for at least half-an-hour, in order to remove the platelets. The oxalate plasma so obtained must be absolutely clear and free from any reddish tinge. To obtain the serozyme, one-tenth volume of 1 per cent. $CaCl_2$ was added to a portion of the plasma, which was then allowed to stand for a quarter of an hour, preferably at 37° C.; possibly a few more drops of $CaCl_2$ would be required to promote clotting. After separating the clot from the serozyme, the latter must be left to stand for some time, as more clot

* *Amer. Journ. of Med. Sciences*, Oct. 1917, p. 533.

might form and have to be removed. For the tests, the serozyme was diluted with five times its volume of normal saline, and 0·5 c.c. was used for each tube. As it must contain neither cytozyme nor thrombin, a control test must be made with serozyme, $CaCl_2$ solution and oxalate plasma; no coagulation should follow, or at least not until the next day. The author found that the quality of serozyme varied on different occasions even when obtained from the same animal. He was able to improve a weak serozyme by allowing it to stand for some hours in 10 times its volume of distilled water; 10 per cent. saline was then added until it contained 0·8 per cent., and it was used in doses of 1 c.c. He found that guinea-pigs, oxen and human beings did not yield serozyme in sufficient amount; the serozyme from rabbits was serviceable, but difficult to procure in the required quantity.

$CaCl_2$ solution.—This was normal saline + 5 per cent. of 1 per cent. $CaCl_2$.

Oxalate plasma.—The mode of preparation is given under serozyme. For use as fibrinogen it must be centrifuged twice, as soon as possible, to remove the platelets. If, in spite of this, small coagula appeared, the plasma might be filtered through cotton wool. When quite clear, it would keep one or two weeks in the ice-chest. For use, 1 part of plasma was diluted with 5 of saline and $\frac{1}{2}$ of 1 per cent. sodium oxalate.

Patient's serum.—This must be well centrifuged, not tinged with blood, and thoroughly inactivated by heating for 1 hour at 56° C., so as to destroy cytozyme.

The test.—0·1 c.c. of the inactivated serum was added to 0·1 c.c. of each dilution of extract, a tube of serum alone and one of extract alone being included as controls. The tubes were well shaken and allowed to stand for $\frac{1}{2}$—1 hour at room temperature. Then to each was added 0·5 c.c. of the serozyme dilution and 1 c.c. of the $CaCl_2$ solution. After thoroughly mixing and leaving for 15 minutes, 1 c.c. of diluted oxalate plasma was added. If the serozyme was good, the control would coagulate after 1, 3 or 5 minutes: "negative sera postponed the time of coagu-
" lation for some minutes, while positive sera definitely inhibited
" coagulation." The author stated that if "auto-coagulation" occurred this had nothing in common with the "auto-inhibition" sometimes found in the Wassermann reaction, "for, if a second
" specimen of the same patient's blood was taken and more
" carefully handled, the serum could always be used."

Like Brandt, the author gave protocols showing that "not
" every dose of cytozyme affords serviceable figures, for,
" proportionate to the strength of the serozyme, &c., sometimes
" the larger, sometimes the smaller, amounts of cytozyme
" furnish the determining factors for a diagnosis."

When in doubt about his serozyme, he made a preliminary experiment, of which the following is an example with a very weak serozyme :—

——	Without Serum.	+ Serum.	Weak + Serum.	— Serum.
Extr. 1:40 - - -	2	15	7	4
„ 1:120 - - -	4	30	20	6
„ 1:240 - - -	6	45	30	15
Serum without extract -	fl.	fl.	fl	fl.

Here diagnosis was to be based on the results with 1 : 120 extract.

On comparing the W.R. and the C.R. with 500 sera, Uemura found that, not excluding cases where merely the strength of the reactions differed, the reactions agreed in 92·75 per cent. In the remainder the history was frequently in accord with the C.R. Only eight cases which were positive both clinically and to the W.R. gave a negative C.R., and in these he suggested that the condition of the specimen might have been at fault. Of the 269 cases which were regarded clinically as probably lues, the C.R. gave 173 positives, but the W.R. only 154. Uemura stated that the technique of the W.R. at the Zurich Institute had been altered since Brandt did his comparative work there, and had been made 10 per cent. more sensitive. The new method was based on a proposal of Fraenkel's. Taking advantage of the fact that hæmolysis took place more quickly in the tubes containing negative sera + extract than in the controls containing extract alone, they employed the extract in an auto-inhibitory dose, and added to it relatively large amounts of serum (up to 0·5 c.c.) ; negative sera prevented inhibition, whilst positive sera increased it. He found that the C.R. was not made more sensitive by using more than 0·1 c.c. of serum, and that larger amounts were disadvantageous, owing to the increased delay of coagulation attributable to normal sera. He often noticed that when a serum gave a slightly + W.R. and a − C.R. it was blood-tinged. He showed, by adding 1−3 drops of washed human 5 per cent. corpuscles to active serum and then heating the mixture, that this was because the blood diminished the inhibitory action of the serum. The experiment did not succeed if the blood was heated separately and added to inactivated serum.

His general conclusion was that the C.R., properly performed, was "highly characteristic in the case of syphilitic serum," and in many cases "distinctly superior to the W.R."

Sordelli and Fischer.

Sordelli and Fischer (1917)* compared the C.R. and W.R. on 210 patients at Buenos Aires, following the method of Hirschfeld and Klinger. In 193 cases (93·2 per cent.) the results of the two tests were in agreement; 5 cases gave a positive C.R. and a negative W.R.; 9 gave a negative C.R. and a positive W.R.; and in 3 the comparison could not be made as the serum was auto-inhibitory.

Freund.

Julius Freund (1918)† compared the reactions of (1) von Dungern, (2) Bruck, (3) Meinicke, and (4) Hirschfeld and Klinger. He agreed with the pathologists who regarded the first two tests as unspecific and unreliable, and, after much investigation, he had come to the same conclusion about the third. But he approved of the coagulation test. It was simpler and cheaper than the Wassermann test; in cases of syphilis it gave more positives than the W.R. performed with normal extract, and its specificity was established by control tests. The following are his data:—

Clinical Diagnosis.	No. of Tests.	W.R. +	C.R. +	W.R. −	C.R. −	Percentages. W.R. +	Percentages. C.R. +
Lues I. - - -	61	41 3±	44 5±	17	12	72	80
Lues II. - - -	71	65	67 2±	6	2	91·5	97
Lues II. recens - -	113	96 1±	99 2±	16	12	86	89
Lues III. - - -	12	8	8	4	4	67	67
Lues latens - - -	83	36 3±	41 8±	44	34	47	59
	340	253	276	87	64	72·7	78·4

Only in 2 cases (Lues II.) did he get a positive W.R. and a negative C.R.; these results were confirmed by repetition. He often found that the + C.R. came out earlier than the + W.R.

As controls he took sera from 36 normal persons and from the following cases:—soft sore, 60; typhus, 14; malaria, 10; pneumonia, 14; recurrent fever, 10; phthisis (advanced), 8. Only one of these gave a + C.R., the W.R. also being +; the case was eventually thought to be latent syphilis.

* *Deutsch. med. Wochenschr.*, p. 326.
† *Deutsch. med. Wochenschr.*, p. 1078.

Relative importance of Physical and Biological Properties of Syphilitic Serum.

I have called attention to two opposite views as to the nature of the Wassermann reaction. One is that it is a biological test, like admittedly specific tests, for a special substance contained in syphilitic serum. The other is that it is a clumsy and needlessly complicated way of demonstrating a physical property of such serum. Another alternative is that a compromise may be effected between these two views.

Evidence of Antibody in Syphilitic Serum.

Toyosumi (1909)* regarded it as generally admitted that the W.R. was a colloidal precipitation between cellular elements and elements in the serum. But this fact did not prove that the W.R. bore no resemblance to specific immunity reactions, because all reactions between specific antigen and antibody were colloidal. Was the W.R. a reaction between antigen and a corresponding antibody? In answer to this question, Toyosumi investigated various normal tissues and ascertained whether they behaved like antigen in fixing the material peculiar to syphilitic sera.

The fresh organs of normal guinea-pigs were rubbed up in a sterile mortar and washed three times with normal saline; the supernatant fluid was poured off and liquid adhering to the tissue was removed with filter paper. Then the syphilitic serum, inactivated at 56° C. for half-an-hour, was added in the proportion of 1 c.c. to 1 gm. of emulsion. After one hour at 37° C. the organ emulsion was removed by the centrifuge and the serum was treated, as before, with fresh organ emulsion. After this second treatment the clear serum was put through the W.R. Other experiments were made with bacteria, either fresh or killed by heating at 65° C. for one hour, 0·8 c.c. of serum being used for one agar culture.

It was found, with two exceptions, that the Wassermann substance in syphilitic sera had been adsorbed by certain tissues. In order of potency, heart was the strongest, then followed liver and kidney. Brain and spleen were much weaker and often negative; leucocytes and red blood corpuscles were completely inactive. This order corresponded with the relative utility of these tissues in furnishing suitable extracts for the ordinary Wassermann antigen. Toyosumi's explanation of the two exceptional sera was that they were particularly strong positives, and the treatment with organ emulsion had not sufficed to remove the whole of the Wassermann substance.

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* *Centralbl. f. Bakteriol.*, Orig. LI., p. 601.

In the control tests bacteria failed to remove the Wassermann substance; and, when bacterial antisera were compared with syphilitic sera, it was found that the bacterial antibody was removed by the appropriate antigen but not by the organ emulsions which removed Wassermann substance.

Toyosumi recognised that an interaction between two colloids which led to the disappearance of each was not necessarily an antigen-antibody reaction; but, he considered, the strongly selective action between syphilitic sera and certain parenchymatous organs resembled a true antigen-antibody reaction so closely as to afford presumptive evidence that Wassermann substance behaved like an antibody.

The Significance of Precipitation.

Sachs and Georgi (1918)* proposed the following sedimentation test for syphilis and compared it with the W.R.

One c.c of 1 : 10 inactivated patient's serum was mixed with 0·5 c.c. of 1 : 6 cholesterinised alcholic extract of bovine heart. Three controls were used:—(*a*) a positive and a negative serum; (*b*) a mixture of each patient's serum with alcohol diluted 1 : 6 in saline; (*c*) 0·5 c.c. of the extract dilution mixed with 1 c.c. of saline.

The tubes were well shaken and kept first for two hours in the incubator and afterwards for 18–20 hours at room temperature. Readings were then taken with the agglutinoscope.

Negative sera were translucent or only faintly opalescent. Tubes showing more evidence of turbidity, which raised a doubt as to the homogeneity of the mixture, were recorded as doubtful. Positive reactions showed bright granules on a dark ground. When there was definite formation of such granules, the result was recorded as +; more marked flocculation was designated ++ or +++.

Crude alcoholic extracts were not satisfactory because they were not sufficiently susceptible to flocculation. They therefore used cholesterinised extracts and standardised them for the test with a large number of syphilitic and non-syphilitic sera. In preparing the extract, they extracted 1 gm. of moist heart-muscle with 5 c.c. of alcohol. To this extract, both concentrated and diluted with various amounts of alcohol, were added graded doses of 1 per cent. alcoholic cholesterin solution, in order to determine the optimum dilution and the optimum amount of cholesterin. As for the W.R., it was important to understand that the activity of the extract depended " not on a single lipoid " but on the interaction of several lipoidal substances, and not " on their absolute but on their relative concentrations." The composition of their extracts generally worked out at about 100 c.c. crude extract + 200 alcohol + 13·5 c.c. of 1 per cent.

* *Med. Klinik*, p. 805

alcoholic cholesterin solution; but each new extract had to be tested separately for optimum dilution and optimum quantity of cholesterin, these two factors being inversely proportional to each other.

The method of diluting the extract was important. Quick dilution gave the least active extract. On the other hand, if the dilution was made too slowly, by the fractional method, it might, after standing some time, lose its homogeneity; hence the necessity for employing an extract control in the test. They recommended the following method of diluting the extract. To the measured quantity of alcoholic extract an equal volume of saline was added quickly. The flask was then held as nearly as possible in the horizontal position and was gently rotated. Then four volumes of saline were quickly added. The six-fold dilution thus obtained was opalescent but clear and translucent.

In 2,770 cases, mostly suspected of syphilis, they found that their sedimentation method agreed with the W.R. in 94·94 per cent., and in 5·06 per cent. the results were divergent; in 3·18 per cent. the sedimentation method was stronger, or was positive as opposed to negative, and in 1·88 per cent. it was weaker, or was negative as opposed to positive.

The authors thought that their sedimentation method should receive further trial before arriving at a decision as to its utility.

This method has already been tried by other investigators, and some data have been published which appear to show that it is as reliable as the German method of conducting the W.R.

Two Factors in the W.R.

Meinicke (1917)* published a note on sero-chemical tests for syphilis in which, as opposed to Bruck's views, he maintained that the result of the W.R. depended on two factors, viz.:—

(*a*) The serum constituents which were characteristic of syphilis were bound in a relatively firm complex with the antigen lipoids. "This alone is the essential part of the reaction."

(*b*) There were conditions which might modify the result of the reaction but did not express the essential nature of the reaction. Thus various artificial means might be used to alter a normal serum so that it became autoinhibitory or gave a + W.R. These results were due to changes in the quantitative relations and the degree of dispersity of the globulins, with consequent changes in their precipitability. Quantitative increase and more ready precipitability of the globulins also accounted, at least in part, for the non-specific results of the W.R. in certain cases of febrile disease. For the same reasons the reactions in secondary syphilis were particularly

* *Münch. med. Wochenschr.*, p. 1464.

strong, because the positive reaction due to (*a*) was intensified by the increased quantity and precipitability of the globulins which were characteristic of this stage of the disease. "Thus " the differences in the precipitability of the serum-globulins " might modify the result of the W.R., but were not themselves " the essential part of the reaction."

In a later note published in the same year,* Meinicke objected to the view of Herzfeld and Klinger that the affinity between extract and the globulins of syphilitic serum was on all fours with the lability of globulins when exposed to the precipitating action of certain chemicals. In his experience, the precipitability of globulins by chemical means was in no way parallel with their avidity for extract lipoids. "Easily " precipitated sera have often not the slightest affinity for " extract and *vice versa*." The essential character of syphilitic serum was the presence of new chemical constituents, peculiar to syphilitics, which, "independently of their chemical precipi- " tability, possess a particular affinity for extract lipoids." The problem to explain was not the precipitability of syphilitic sera but the reason why these new substances made their appearance.

Meinicke (1917)† proposed two new methods for the diagnosis of syphilis by precipitation tests.

1. *The Water Method.*—To 0·2 c.c. of serum, inactivated at 55° C. for one hour, he added 1·5 c.c. of antigen (the German official antigen), diluted with distilled water. The appropriate degree of dilution had to be determined for each antigen; with some it was found to be 1 : 12. The tube was then shaken well and incubated at 37° C. for one hour. He then added 2·5 c.c. of distilled water, previously warmed to 37° C. (In determining the quantities of antigen and water he aimed at slightly exceeding the optimum for each.) The tube was again shaken and incubated for about 16 hours. Readings were then taken as for an agglutination test. Negative sera were clearly flocculated. Positive sera showed a very characteristic opalescent, bluish-grey turbidity, which was somewhat translucent and free from flocculi. Flocculi were absent in all positive cases, even with weak positives. When he wanted to grade his positives he used a second tube containing less water, *i.e.*, favouring flocculation, and thus obtained varying degrees of flocculation with his weak sera.

2. *The Salt Method.* — This method depended on the behaviour of serum precipitates towards saline solutions. To 0·2 c.c. of serum, inactivated at 55° C. for $\frac{1}{4}$ hr., he added 2 c.c. of antigen diluted 1 : 8 in distilled water, shook well and allowed to precipitate for about 16 hours at 37° C. All sera, both normal and syphilitic, then showed a well marked, densely flocculated deposit. The next step was to run in 1 c.c. of

† *Münch. med. Wochenschr.*, p. 1644.
‡ *Berl. klin. Wochenschr.*, p. 613.

distilled water down the side of the tube, and to shake up the contents so as to get as uniform a suspension as possible. He then added cautiously 1 c.c. of 2·5 per cent. saline, allowing the fluid to run down the side of the tube; shaking was to be avoided and great care was to be taken not to cause any violent agitation. The tubes were incubated for one hour at 37° C., and readings were then taken. With negative sera the deposit was dissolved or on the point of dissolving; with positives it remained in flocculi, which varied in density according to the strength of the reaction.

In carrying out his technique, Meinicke laid great stress on the importance of slow dilution of the antigen. This, he remarked, was equally important for the W.R., if constant results were to be obtained. He placed his antigen in a measuring cylinder and ran in the water from a burette, drop by drop, and with a regular flow, so calculated that it took about $1\frac{1}{2}$ minutes to deliver a quantity of water equal to the quantity of extract, whatever that quantity might be. In this way a permanently turbid dilution was obtained.

Shortly afterwards, Meinicke published a second article,* in which he claimed that his salt method could be used as a general means for the detection of a specific antigen-antibody combination. He considered that there was an absolute parallel between complement-fixation and fixation of lipoid, and that this was demonstrated by the fact that antibody, through its union with specific antigen, was capable of altering organ lipoids in just the same way as it altered complement.

Working first with glanders, he made an alcoholic extract of horse heart, and diluted this with water, in such proportion that all horse sera, after treatment overnight, were precipitated with certainty. These precipitates, in the case of normal sera, were soluble in particular percentages of saline. The principle of the test was to bring together serum, extract of glanders bacilli, and descending doses of the lipoid extract; when the serum contained glanders antibody, the precipitate which had formed was much less readily dissolved in saline than the precipitate obtained with normal serum.

He also found the method successful for the demonstration of dysentery antibodies in convalescents, for typhoid antibodies, and for the specific antibodies obtained by immunising rabbits with protein from guinea-pigs, horses, and sheep. The method also served as a test for specific antigens. He found it best to prepare his lipoidal extract from organs of the same species as the blood to be tested.

As regards details of technique, the lipoidal extract was diluted 1 : 8 by the slow method, and specific antigen was added in amount corresponding to its titre. To 0·2 c.c. of the serum to be investigated was added an average amount of 1 c.c.

* *Berl. klin. Wochenschr.*, p. 1208.

of the mixture of lipoidal extract and specific antigen. After incubation overnight, the deposit was gently shaken until a uniform, coarsely granular suspension was obtained. Then he added 1 c.c. of saline, the strength of which had been determined by titration with undoubtedly negative sera, incubated at 37° C. for one hour, and read the results as in an agglutination test. In the positive tubes the flocculi persisted; in the negative they were dissolved.

In a later article (1918)* he claimed that his salt test should rank with the W.R. as a valid test for syphilis, and might serve as a control to the latter.

W. Gärtner (1918)† compared the Bruck and the Wassermann reactions in a series of over 28,500 cases. In the Bruck test which he used, a mixture was made of 0·5 c.c. patient's serum, 2 c.c. distilled water, and 0·3 c.c. of 25 per cent. HNO_3; the deposit thus formed was completely dissolved by 16 c.c. of water in the case of healthy patients, but with syphilitics an undissolved residue was left. In addition to using 0·3 c.c. of HNO_3, which was the amount prescribed by Bruck, Gärtner also used, in from 700 to 1,000 cases, ascending or descending quantities, in order to determine the limit at which a deposit was just retained or just dissolved. Whilst agreeing with other observers that the Bruck test was not specific for syphilis, Gärtner thought that useful information might be obtained by comparing the Bruck with the Wassermann test in different stages of this disease.

In the following table Gärtner summarised the results of this comparison. + = strong reaction; ± = weaker; ∓ = weak; − = negative.

Stage of Disease.	W.R.	Bruck (with 0·3 HNO_3).	Limit for deposit in c.c of HNO_3.
Early Primary -	−	−	normal
Late Primary - -	± and +	−, later +	0·34 to 0·24
Early Secondary -	∓ (100 °/₀)	+ (100 °/₀)	0·2 to 0·24
Late Secondary -	+	+ and −	0·24 to 0·32
Early Latent - -	+, sometimes −	+, more often −	0·28 to 0·32
Tertiary - - -	+ and −	− and +*	0·28 to 0·34
Late Latent - -	+ and −	−	over 0·32
Metasyphilitic -	+ and −	−	almost normal

* + when there was breaking down of tissues.

Thus, comparing the reactions according to the stage of the disease, there was a general indication that the Bruck positives commenced later than the W.R. positives and disappeared earlier.

* *Münch. med. Wochenschr.*, p. 1379.
† *Centrbl. f. Bakteriol.*, Orig., LXXXII., p. 337.

When, however, Gärtner compared the results of the two reactions during the course of treatment he found that the Bruck remained positive longer than the W.R.

His explanation was that in the secondary and late primary cases the Bruck positives were at first "specific," but in the course of treatment these reactions receded and were replaced by "non-specific" positives. In support of this view he quoted his experience of the relation of calomel abscesses to the Bruck test. On two occasions he noticed that after evacuation of such abscesses and suspension of the treatment by injection the Bruck reaction became negative after a relatively short time. This led him to make further observations, from which he concluded that the processes of infiltration, induced by calomel, led to an increase of the globulins in the plasma, and the positive Bruck reaction was the demonstration of this increase.

Gärtner tested the blood of patients withdrawn before and immediately after infusion with 1—1¼ litres of saline. The W.R. remained unaltered, but the Bruck reaction was weakened by the artificial hydræmia, *i.e.*, it took a greater concentration of HNO_3, acting for the usual time of 10 minutes, to produce a permanent deposit. This result he attributed to a diminished concentration of the globulins; it seemed less tenable to postulate that dilution had diminished the susceptibility of the globulins to precipitation.

The Bruck test simply showed that tissue disintegration was going on, and this might or might not be due to syphilitic infection; the W.R. was specific because it demonstrated Wassermann antibody.

Comments.

Meinicke's suggestion that the W.R. depends on two factors seems interesting and helpful. I do not think that agreement has yet been reached as to the value of his precipitation tests. The method of Sachs and Georgi seems to be considered more attractive.

The Influence of Cold Fixation on the Wassermann Reaction.

Laboratory Data as to Value of Cold Fixation for Diagnosis.

Jacobsthal (1910)* investigated the effect of cold fixation on the adsorption of complement in the Wassermann reaction.

In performing the test he used 0·05 inactivated serum, 0·05 guinea-pig complement, 0·5 of 5 per cent. sheep's corpuscles with twice the lytic dose of amboceptor, and graduated doses of extract, each ingredient being made up to 0·5 c.c. Serum, extract, and complement were mixed in each

* *Münch. med. Wochenschr.*, p. 689.

of three tubes; the first was kept for $1\frac{1}{2}$ hours at 4° C.; the second was kept for $\frac{3}{4}$ hour at this temperature and then for $\frac{3}{4}$ hour at 37° C.; the third was incubated for $1\frac{1}{2}$ hours at 37° C. After adding amboceptor and corpuscles, the tubes were incubated for 2 hours and were read after 18 hours.

By comparison of 200 sera he found that cold fixation gave sharper results and was preferable, about 2 per cent. more positives being obtained. According to the protocols, positive reactions were rather stronger in the first tubes than in the second tubes, where exposure to 4° C. only lasted for $\frac{3}{4}$ hour.

Guggenheimer (1911)* also compared fixation at 37° C. (incubator) and at 0° C. (tubes kept in dishes filled with finely broken ice) for $1\frac{1}{4}$ hours.

He used decreasing doses of alcoholic extract of syphilitic liver, 0·025 inactivated serum, 0·025 guinea-pig serum, and 0·25 corpuscles sensitised with a dilution of amboceptor which would give from 3 to 4 doses, each of the five ingredents being made up to 0·25 c.c. Readings were taken after 2 hours incubation.

He found that at 0° C. the anticomplementary action was always more or less stronger than at 37° C., and he was therefore particularly careful about the extract control, only counting results as positive when he was satisfied that this was free from objection. Results :—

	+ at 37° and 0°.		− at 37° and 0°.	+ at 37° only.	+ at 0° only.
Equally strong - -	186	255	348	8	12
Stronger at 37° - -	32				
Stronger at 0° - -	37				

Summary :—

	+	−	
at 37°	263	360	in 623 cases.
at 0°	267	356	

In his opinion, the above results did not justify replacing the warm by the cold method but suggested the utility of doing parallel experiments with each.

Altmann and Zimmern (1912)† compared the effects of cold ($1\frac{1}{4}$ hours in the ice-chest) and warm fixation on 1902 sera. Their technique was essentially the same as that of the Frankfurt Institute, only they titrated complement as well as amboceptor and used $2\frac{1}{2}$ doses of each. They adopted Sachs's antigen, but generally tested each serum with graduated doses of two or three extracts, one of which was prepared from a syphilitic liver.

With 1,610 sera both methods yielded completely identical results (1,025 negative and 585 positive). Of the remaining

* *Münch. med. Wochenschr.*, p. 1392.

† *Arch. f. Dermat. u. Syph.*, Orig., III., p. 837.

292 sera, 216 were positive by the warm method and 247 by the cold, giving a total of 31 more positives by the cold method in 832 positive sera. They thought the cold method highly valuable for cases in the latent period or during treatment.

In 270 "normal" sera from patients with fever or wasting disease, the cold method gave only three positives, and in each of these earlier infection with syphilis could not be excluded.

The above tests were made with inactivated sera; with active sera the cold method was found unreliable. Out of 929 active sera, 303 were alike by both methods, 512 showed slight differences, always in favour of the warm method, and there were absolute differences in 114, which were positive by the warm method only.

They thought differences in the results of cold and warm fixation were probably attributable to properties in the patients' sera.

Altmann (1913),* employing the same method as before, but using exclusively a cholesterin antigen (guinea-pig or ox heart), tested 1,378 additional sera. 224 of these were negative. Of the remaining 1,154 sera, 783 (67 per cent.) were equally positive by both methods; 160 were obviously different, 70 (6 per cent.) being stronger by the warm method, and 90 (8 per cent.) stronger by the cold; in 211 cases the results were absolutely different, 84 (8 per cent.) reacting only by the warm method, and 127 (11 per cent.) only by the cold. He found that in the primary stage the warm method surpassed the cold by $27\frac{1}{2}$ per cent., and in the early secondary stage by $2\frac{1}{2}$ per cent., whilst in the late stages the cold method surpassed the warm by $17\frac{1}{2}$ per cent

Leredde and Rubinstein (1914)† compared cold fixation with the ordinary method of conducting the Wassermann test in 1,448 cases (1,338 sera and 110 cerebro-spinal fluids). In using the cold method, each ingredient was separately cooled before mixing, the temperature in the interior of the tubes being about 6° C.; the mixture of serum, complement, and antigen was kept at this temperature for $1\frac{1}{4}$ hours. Sensitised corpuscles were then added, and final readings were taken after an hour's incubation.

Of the sera, 982 could be classified clinically as follows:—primary syphilis, 210; secondary, 253; tertiary, 180; general paralysis of the insane, 176; tabes, 118; latent syphilis, 45. Out of the total 982 there were 25 where warm fixation gave a positive result and cold fixation a negative; in 9 of these the difference was absolute, and in the remaining 16 it was only a difference in degree of hæmolysis. With 151 sera cold fixation gave a stronger reaction than warm fixation; in 77 of these the difference was absolute, and in 74 it was merely quantitative. Omitting the last, there were thus 77 cases where cold fixation alone gave a diagnosis in conformity with the clinical condition,

* *Arch. f. Dermat. u. Syph.*, Orig., CXVI., p. 871.
† *C.R. Soc. de Biologie*, LXXVI., p. 485.

the majority being late stages of syphilis and treated cases. Moreover, in the authors' opinions, a sharp positive obtained by the cold method strengthened the diagnosis where fixation at 37° C. gave only a weak positive.

The above data referred to tests on inactivated sera. At the same time the authors examined the fresh sera by both methods. In the majority of cases, positives by the cold method with heated serum corresponded with positives obtained with fresh serum, both by the cold and the warm method; but sometimes there was complete disagreement, the serum, both fresh and inactivated, being negative by the warm method and positive by the cold.

The sera of 221 non-syphilitic persons were equally negative by both cold and warm methods.

In the examination of 110 spinal fluids, no difference was revealed between the two methods.

In none of the cases examined did the authors find that anticomplementary action of either serum or antigen would suffice to explain differences in results obtained by the use of different temperatures.

J. W. Smith and W. J. MacNeal (1916)* made 496 tests upon 477 patients by three different methods, the differences being the use of (1) cholesterinised antigen at 37° C., (2) alcoholic extract without cholesterin at 37° C., (3) the latter antigen at 8° C.

The antigens used were alcoholic extracts of human, ox, and guinea-pig heart (10 gms. of muscle in 100 c.c. of absolute alcohol), and the same extracts reinforced by 0·4 per cent. cholesterin. "Just before use, each antigen was diluted "according to previous titrations with from 10 to 30 volumes "of salt solution to produce a fine emulsion, of which 0·1 c.c. "would be the proper dose for each tube."

The patients' sera were inactivated at 56° C. just before use.

Complement was 10 per cent. fresh guinea-pig serum.

Sensitised cells were prepared by mixing 5 per cent. cells (sheep) with an equal volume of amboceptor of such strength that 0·1 complement + 0·2 corpuscles + 0·2 diluted amboceptor + 0·5 saline gave complete haemolysis in 15 minutes at 37° C. The requisite titrations of the hæmolytic system were made before each test.

The following table shows the scheme for the tests at 37° C.:—

——	Tube 1.	Tube 2.	Tube 3.	Table 4.
Patient's serum - -	0·04	0·02	0·04	0·08
Cholesterinised antigen -	0·1	0·1		
Simple antigen - - -			0·1	
Complement - - -	0·2	0·2	0·2	0·2
Saline - - - -	0·3	0·3	0·3	0·3

* *Journ. of Immunology*, II., p. 75.

After 1 hour at 37° C., 0·4 of the suspension of sensitised cells was added. Readings were taken when the serum and antigen controls showed complete hæmolysis, usually in 15—30 minutes.

For the test at 8° C. the following scheme was employed:—

	Tube 1.	Tube 2.	Tube 3.
Patient's serum - - - - -	0·04	0·02	0·08
Simple antigen - - - - -	0·1	0·1	
Complement - - - - -	0·2	0·2	0·2
Saline - - - - - -	0·3	0·3	0·3

The tubes were kept in the refrigerator at least four hours, and usually overnight. 0·4 c.c. sensitised cells was then added and the lower part of the tubes was immersed in a water-bath at 40° C. The readings were taken when the serum and antigen controls showed complete hæmolysis, usually in 10—15 minutes.

The authors classified their results as follows:—

"Positive" = fixation ranging from complete to "about 50 per cent."; "doubtful" = "about 25 per cent. fixation" or less; negative = no fixation.

I.—127 tests on 110 known syphilitics, of whom the majority were under treatment, and 50 were free from manifestations of syphilis at the time of the test.

	Percentage positive.	Percentage doubtful.	Percentage negative.
Cholesterin antigen at 37° C. -	58·2	7·9	33·8
Simple „ „ -	32·2	0·8	66·9
„ „ „ 8° C. -	77·1	4·1	18·8

II.—44 tests on 43 persons probably syphilitic.

	Percentage positive.	Percentage doubtful.	Percentage negative.
Cholesterin antigen at 37° C. -	65·9	4·6	29·5
Simple „ „ -	36·3	2·4	61·3
„ „ „ 8° C. -	75	0	25

III.—60 tests on 59 patients probably not syphilitic.

	Percentage positive.	Percentage doubtful.	Percentage negative.
Cholesterin antigen at 37° C. -	40	15	45
Simple „ „ -	1·6	0	98·3
„ „ „ 8° C. -	5	5	90

IV.—Tests on 265 non-syphilitics.

Results negative throughout by all three methods.

The authors concluded that the last method was more sensitive than the other two, and that a positive result obtained by it was much more trustworthy evidence of syphilis than a positive result with a cholesterinised antigen.

J. W. Smith and W. J. MacNeal (1917)* compared the action of three antigens at 37° C. for 1 hour and at 8° C. for 4 hours on 501 sera from 457 patients. The three antigens were (1) alcoholic heart extract (Alc.), (2) the same with the addition of cholesterin (Chol.), and (3) Noguchi's acetone-insoluble preparation (Nog.). They were all prepared from ox-heart. 120 gms. were extracted with 1,200 c.c. of absolute alcohol at 37° C., with occasional shaking, for two weeks. The preparation was then filtered through paper until clear. For the cholesterin antigen, 0·4 gm. of cholesterin was added to 100 c.c. The mixture was refrigerated overnight and again filtered.

Antigen (1) was not hæmolytic and not anticomplementary in the maximum quantities tested. The smallest amount necessary for complete fixation was 0·05 of a 10 per cent. emulsion at 37° C., and 0·2 of a 1 per cent. emulsion at 8° C., the tests being made with 0·1 of a 1 : 5 strongly positive serum in a total volume of 1 c.c. Antigen (2) was not hæmolytic at either temperature in the maximum quantity tested. It was anticomplementary in doses of 0·4 c.c. at 37° C. and at 8° C. The antigenic titre was 0·2 of a 1 per cent. emulsion at 37° C. and 0·1 of the same at 8° C., 0·2 of 10 per cent. complement being used.

In their hæmolytic system the unit of cells was 0·2 of 5 per cent. sheep's corpuscles and the unit of complement was 0·1 of 1 : 10 guinea-pig serum. The unit of amboceptor (rabbit) was determined each time, and was always about 0·1 of 1 : 1000; this, with 1 unit of complement, produced complete lysis of 1 unit of cells in 15 minutes at 37° C. In the tests, 2 units each of amboceptor and complement were used. This system the authors regarded as "quite sufficiently loose to obviate very "largely the danger of non-specific fixation"; yet they found reactions in 16 of their 501 tests (3·2 per cent.), which they felt inclined to regard as false positives (*see* Table II.c *below*).

The sera were always heated at 56° C. for half-an-hour just prior to use.

The tests for incubation at 37° C. and 8° C. were always exact duplicates. Four tubes were used for each, the contents being:—

(1)	Serum,	0·04;	antigen	Chol.,	0·1;	complement,	0·2;	saline to 0·6.
(2)	„	0·04;	„	Alc.,	0·1;	„	0·2;	„
(3)	„	0·04;	„	Nog.,	0·1;	„	0·2;	„
(4)	„	0·08;	———		;	„	0·2;	„

* *Journ. of Infect. Diseases*, XXI., p. 233.

After incubation, 0·4 of sensitised cells was added. The tests were read when the control tubes showed complete hæmolysis, usually in 15—30 minutes.

Results:—

I.—92 tests on 80 known syphilitics, mostly treated.

Extract.	Temp.	Percentage Positive.	Percentage Doubtful	Percentage Negative.
	°			
Chol. - - -	37	52·1	3·2	44·7
Alc. - - -	37	26	1	73
Nog. - - -	37	31·5	0	68·5
Chol. - - -	8	76	3·2	20·8
Alc. - - -	8	64·1	4·3	31·6
Nog. - - -	8	46·7	3·2	50·1

IIA.—27 tests on 24 evident syphilitics (lesions typical).

Extract.	Temp.	Percentage Positive.	Percentage Doubtful.	Percentage Negative.
	°			
Chol. - - -	37	92·5	0	7·5
Alc. - - -	37	66·6	3·8	29·6
Nog. - - -	37	77·7	0	22·3
Chol. - - -	8	100	0	0
Alc. - - -	8	92·5	0	7·5
Nog. - - -	8	88·7	3·8	7·5

IIB.—25 tests on 20 probable syphilitics.

Extract.	Temp.	Percentage Positive.	Percentage Doubtful.	Percentage Negative.
	°			
Chol. - - -	37	84	0	14
Alc. - - -	37	48	8	44
Nog. - - -	37	68	4	28
Chol. - - -	8	92	4	4
Alc. - - -	8	88	8	4
Nog. - - -	8	80	8	12

Summary of I, IIA, and IIB (144 tests on 124 patients certainly or probably syphilitic).

Extract.	Temp.	Percentage Positive.	Percentage Doubtful.	Percentage Negative.
	°			
Chol. - - -	37	65·3	2·1	32·6
Alc. - - -	37	37·5	2·8	59·7
Nog. - - -	37	46·5	0·7	52·8
Chol. - - -	8	83·3	2·8	13·9
Alc. - - -	8	73·6	4·2	22·2
Nog, - - -	8	60·4	4·2	35·4

IIc.—16 tests on patients probably not syphilitic.

Extract.	Temp.	Percentage Positive.	Percentage Doubtful.	Percentage Negative.
	°			
Chol. - - -	37	50	18·8	31·2
Alc. - - -	37	0	0	100
Nog. - - -	37	0	0	100
Chol. - - -	8	31·2	18·8	50
Alc. - - -	8	0	0	100
Nog. - - -	8	0	0	100

III (including IIc).—357 tests on 333 non-syphilitics.

Extract.	Temp.	Percentage Positive.	Percentage Doubtful.	Percentage Negative.
	°			
Chol. - - -	37	2·2	0·8	97
Alc. - - -	37	0	0	100
Nog. - - -	37	0	0	100
Chol - - -	8	1·4	0·8	97·8
Alc. - - -	8	0	0	100
Nog. - - -	8	0	0	100

The authors' conclusions were that the cholesterin antigen at 8° C. "constitutes a more sensitive test for syphilis than "does any of the other methods examined"; but this test, both at 37° and 8°, "is apt to yield non-specific complement-fixation"; and, therefore, "in a diagnostic reaction, fixation "with the cholesterin antigen alone is, at best, of only doubtful "significance." They regarded the simple alcoholic extract at

8° as more sensitive than the cholesterin antigen at 37°, and found no evidence that it gave false positives. They found Noguchi's antigen less sensitive at 37° and 8° than the cholesterin antigen at either temperature, and also less sensitive than the simple extract at 8°, but more sensitive than the last at 37°; there was no evidence that Noguchi's antigen gave false positives.

Fairley and Sullivan (1919)* adopted the following technique for comparing cold and warm fixation. They employed the cholesterinised alcoholic heart extract of Fildes and McIntosh,† taking care that never more than half the anticomplementary dose was used. For the hæmolytic system they used 3 per cent. sheep corpuscles sensitised with 4 M.H.D. of hæmolytic serum. The patient's serum was diluted with four volumes of saline and heated at 55° C. for 20 minutes. The tubes were put up in four rows. The first contained one volume each of antigen, serum, and saline, and one volume containing 3 M.H.D. of complement. The second and third rows were the same, except that the second contained 5 M.H.D. of complement and the third 7 M.H.D. The fourth row was the serum control (serum + saline + 3 M.H.D. of complement). They also used two antigen controls, one containing one volume of antigen and the other two volumes, and also a pooled negative and a pooled positive serum. In the ordinary method, the tubes were incubated for one hour at 37° C. before adding the volume of sensitised corpuscles. In the ice-box method, 4, 6, 8, and 4 M.H.D. of complement were used, the addition of 1 M.H.D. being intended "to compensate for the slightly increased "tendency for fixation of complement by normal sera with this "method." The time employed for cold fixation was always 6 hours and the temperature was 8° C. The following results were obtained:—

I. Untreated Syphilitics.

	Total examined.	Positive by	
		Warm Method.	Cold Method.
Primary	70	44	52
Secondary	62	60	62
Tertiary	80	67	76
Latent	4	3	4
	216	174	194

* *Journ. of R.A.M.C.*, XXXIII., p. 268.
† *Lancet*, Oct. 28th, 1916.

II. Treated Syphilitics.

	Total examined.	Positive by	
		Warm Method.	Cold Method.
Primary - - - - -	109	28	32
Secondary - - - -	129	44	60
Tertiary - - - - -	62	41	51
	300	113	143

In four cases out of 678 in which there was no clinical history of syphilis, slight positives were recorded (absence of lysis in first row). In the remaining cases, negative results were obtained by both methods.

On the strength of the above results the authors recommended the adoption of cold fixation. In their opinion, the advantage of the method was that it increased the time available for the reaction. Complement deteriorated rapidly at 37° C., and therefore long exposure to this temperature was impossible, but at 8° C. they could detect no deterioration, even after 24 hours, or longer. They found that excess of complement absorbed by the ice-box method over and above that absorbed at 37° C. in one hour was 2 M.H.D. for primary syphilis, 5 for secondary, 6 for tertiary, and 5 for treated relapsing syphilis. The complement absorbing power of negative serum + antigen was never more than $\frac{1}{2}$ M.H.D. greater at 8° C. for six hours than at one hour at 37° C.

Theoretical Considerations.

Under this heading I propose to refer to (1) Jacobsthal, (2) Dean, and (3) other investigators, and to quote observations which have a bearing on the principles underlying cold fixation both in the W.R. and in reactions which are admitted to be specific.

(1.)

Jacobsthal (1910)* thought there were two advantages in using cold fixation for the first part of the W.R. Adsorption was increased and consequently anticomplementary action was strengthened; it also appeared that the cold facilitated the precipitation of the extract lipoids.

* *Münch. med. Wochenschr.*, p. 689

In his opinion (1911)* the W.R. depended on a precipitation process. The precipitation was often visible to the naked eye, and it could regularly be demonstrated microscopically with dark ground illumination, which showed that the minute granules and drops of the extract had been changed, in a positive W.R., into large flaky conglomerations.

(2.)

Dean (1912),† for the purpose of studying the mechanism of complement fixation, prepared mixtures of antigen with antiserum which were so diluted that little or no visible precipitate was formed; he then observed the effect of adding fresh guinea-pig serum to these mixtures. As antigens he used normal human serum, normal horse serum, and watery extracts of *B. typhosus;* the antisera were prepared by intravenous injection of rabbits. Many experiments were performed and similar results were obtained in all cases. The addition of complement increased the amount of precipitate, and, when the quantities of antigen and antibody were so arranged that the combination formed no visible precipitate but nevertheless bound complement, the addition of complement caused a precipitate to appear after an interval of 6 to 24 hours. His procedure was to incubate his tubes, with and without complement, for 5 hours at 37° C., and then to keep them overnight at 8° C. before taking the readings. Using the same graded doses of antigen (11 different dilutions) and antiserum (4 dilutions) and comparing precipitation with complement fixation, he found that the relative proportions of antigen and antiserum which favoured increased precipitation in the presence of complement were also the proportions which were suitable for complement fixation.

In explanation of his experiments, Dean suggested that in the interaction between antigen and antibody there was a specific precipitation of the proteids of the antiserum, but, if the antiserum was very small in amount, the precipitated particles would be too small to be visible. If to such a mixture complement was added, it was quite possible that the proteids of the guinea-pig serum were adsorbed by the minute particles of the specific precipitate. The latter would thus be increased in size and become visible as a definite turbidity or precipitate. The fact that no precipitate was visible in the simple mixture of antigen and antibody was therefore no evidence that complement fixation was independent of the formation of a precipitate. On the contrary, his suggestion was that "this adsorption of "the proteids of the guinea-pig serum by the minute particles "of a precipitate is the essential cause of the fixation of "complement."

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* *Zeitschr. f. Immunitätsforschung*, Orig., VIII., p. 107.
† *Journ. of Hygiene*, XII., p. 259.

In a further series of experiments he took fixed quantities of antigen (typhoid extract) and antibody, which yielded a precipitate of 0·01 c.c. in bulk. He then ascertained the amount of precipitate obtained when he had added to the mixture fresh serum (from a guinea-pig, ox, or rabbit) or guinea-pig serum heated for half an hour at 56° C. In each case there was a marked increase in the bulk of the precipitate. There was very little difference between the effects of fresh and heated guinea-pig serum. "This shows that the action of the "normal serum in these experiments does not depend on the "presence of complement."

Dean then made experiments with the euglobulin fraction of guinea-pig serum prepared by the CO_2 method. Using various antigens and antisera, he determined the dilution of antiserum which just failed to produce visible precipitation but was nevertheless effective for the fixation of complement. To this dilution he added a suitable dilution of the antigen together with the euglobulin preparation and found that definite precipitation took place. He inferred that, in his previous experiments, it was the euglobulin fraction of guinea-pig serum which was precipitated by the mixture of antigen and antiserum. "The mid-piece fraction of the complement is probably "fixed or bound by this euglobulin precipitate in precisely the "same manner as it is fixed by the euglobulin precipitate "which is produced by the action of carbon dioxide or weak "hydrochloric acid. The precipitation of the euglobulin of "the guinea-pig serum is probably an essential part of the "mechanism of complement fixation." This precipitation, as he had suggested, might be due to adsorption by the precipitate formed in the interaction between antigen and antibody, but no knowledge was available as to the mechanism whereby a precipitate was formed in a simple mixture of antigen and antibody.

Dean found that, by keeping a precipitating mixture of antigen, antiserum and complement at 0° C., he could demonstrate that the resulting precipitate contained the mid-piece fraction of the complement in an active state. It was necessary to keep the precipitate cold both during and after its formation. "When the experiment was attempted at a higher "temperature it was not found possible to re-suspend the "precipitate in a satisfactory manner. At any rate it was not "possible to demonstrate in it the presence of active mid-piece." He and Ledingham had also found that in obtaining mid-piece by Liefmann's method an ice-cold temperature must be maintained throughout the operation.

The above abstract should be supplemented, on two points, by references to Dean's Horace Dobell Lecture (1917).*

(1) As the precipitates which occurred in mixtures of antigen, antibody, and complement were often not formed until

* *Lancet*, I., p. 45

several hours had elapsed, he adopted the practice of leaving the tubes overnight in the ice-chest. "This practice led to " the observation that these precipitates are formed most " readily at a low temperature and that if, after the precipi- " tate has formed, the tubes are placed in an incubator at " 37° C., the precipitate dissolves and the mixture becomes " once more absolutely clear. On replacing the tubes in the " ice-chest the precipitate re-forms."

(2) With reference to mid-piece he made the following remark:—"It may, however, well be doubted if any such " substance as the mid-piece fraction of the complement exists. " The action of the so-called mid-piece may be entirely due " to the physical state of the adsorbed particles of euglobulin."

Dean (1917)* investigated the influence of temperature on complement fixation reactions with (*a*) normal serum and homologous antiserum, (*b*) bacillary extract and homologous antiserum, and (*c*) alcoholic organ extract and serum from a syphilitic patient.

Two experiments were made with the Wassermann reaction. In the first, fixation at 0° C. (A) was compared with fixation at 18° C. (B). The ingredients for (A) were cooled to 0°, mixed, and allowed to stand for one hour at 0°; they were then incubated for a second hour at 37°. The ingredients for (B) were allowed to acquire room temperature (18°), and were then mixed and allowed one hour at room temperature for fixation; they were then incubated for a second hour at 37°. On adding corpuscles and hæmolytic serum, it was found that more complement had been fixed in set A than in set B. In the second experiment the necessary ingredients were divided into three sets and exposed to temperatures of 0°, 14° (room), and 37°. After one hour the ingredients were mixed. Set A was left for ½ hour at 0°, and set B for ½ hour at 14°. Sets A and B were then incubated for ½ hour at 37°. Set C was incubated for 1 hour at 37°. It was found that more complement was fixed at 0° than at 14°, and more at 14° than at 37°.

Similarly it was found that more complement was fixed at 0° than at 37° with mixtures of antibody and antigen (normal serum or bacillary extract), but the maximum fixation was attained more slowly at 0° than at 37°. In certain experiments the reaction was complete at 37° in ½ hour, while at 0° considerable progress was made after the expiration of the second hour. Comparing results at the end of 1 hour, the advantage was on the side of fixation at 0°. The time required for maximum fixation depended on the proportions of antigen and antibody. If these were optimal, complement was rapidly fixed, but the reaction was retarded by relative excess of either antibody or antigen. When antigen, antiserum, and complement were mixed, the euglobulin of the guinea-pig serum was

* *Journ. of Path. and Bact.*, XXI., p. 193.

adsorbed by the particles of the precipitate. The formation of this adsorption compound, which was favoured by keeping the mixture at a low temperature, was regarded by Dean as an essential part of the mechanism of complement fixation. He considered that complement was fixed during the earliest stages of the reaction between antigen and antibody, and that little or no complement was fixed after a visible precipitate had been formed. Thus the influence of cold in delaying the formation of a precipitate led to increased fixation of complement.

In the W.R., is the method which gives the maximum inhibition of complement necessarily the most selective for "Wassermann substance?"

Thomsen and Boas (1913)* found that, in investigating the influence of temperature on complement fixation in the Wassermann reaction, it was important to bring the liquids to the required temperature before mixing. In their research they used the Copenhagen method, with colorimetric index, and compared temperatures of 0°, 11–12°, 16–18° (room), and 37° C.

To illustrate the rapidity with which fixation took place they recorded the following experiment :—

Quantity of Serum in c.c.	Duration, in Minutes, of Fixation at 18° C., of Complement, Extract, and Serum, and resultant Percentage of Hæmolysis.					
	0′.	2′.	5′.	10′.	30′.	60′.
0·1	100	0	0	0	0	0
0·05	100	16	0	0	0	0
0·025	100	70	20	14	4	0
0·012	100	90	70	70	25	18
0·006	100	100	100	100	90	45
0·003	100	100	100	100	100	100

Comparing the relation between temperature and binding capacity, in one hour, they found that much the commonest condition was a steady rise of fixation from 0° to a maximum at 18° with progressive diminution as the temperature was increased up to 37°; sometimes the maximum was reached at 18° and persisted up to 37°; and sometimes the maximum was attained at 0°, was maintained up to 18°, and then progressively diminished as the temperature was raised to 37°. Occasionally, fixation was equally good at all the temperatures tried, and, more rarely, it was slight at 0° but progressively increased to 37°, either in a straight line or in a curve with its convexity upwards.

Their general conclusion was that 16–18° was usually the optimum temperature for rapidity of fixation, and that there was no practical advantage in employing fixation at 0°. They

* *Zeitschr. f. Immunitätsforschung*, Orig., XVIII., p. 516.

never found sera which attained a maximum at 0° and decreased progressively at 11°, 16–18°, and 37°. Nor did they find any correlation between optimum temperature of fixation and type, phase, or stage of syphilitic infection. In tests on 200 non-syphilitic sera they found no instance where a positive result was obtained with any of the temperatures employed. No evidence was found that any of the fixation which took place at 18° was reversible at 37°.

At their Institute they made it a rule to fix for ¾ hour at 16–18° and then for ¾ hour at 37° C. before adding corpuscles and amboceptor.

In criticising experimental data it is generally easy to discover that the results might have been different if the technique had been altered. Here, for example, one might readily point out that, if the experiments had been continued for longer than one hour, there would have been a much better chance of finding instances where optimum fixation was obtained at 0° C.

Nevertheless, the data actually obtained are of interest, and I think that three of the main results suggest some comment.

(1) The protocol shows that fixation of a certain amount of complement was rapid, at 18° C., when sufficient sensitising serum was employed. Perhaps the rapid disappearance of complement was here due to chemical action as well as to adsorption. The postulate that it was due to adsorption alone would make it necessary to explain why at 0° C., a temperature particularly favourable to adsorption, removal of complement is relatively slow. The readiest explanation would probably be that though 0° C. is favourable to adsorption, the formation of the requisite adsorbing complex is slow at this temperature.

(2) The temperature for maximum fixation (in one hour) was most commonly found to be 18° C. This may be explained by assuming (*a*) that at 37° C. complement acts more rapidily than at 18° C. and destroys the anticomplementary power of the antigen-antibody complex before all the complement is used up, and (*b*) that at 0° C. complement acts more slowly than at 18° C., and therefore less is used up in the given time.

(3) Various exceptions were noted to the common rule that 18° C. was the optimum temperature for fixation in one hour. These irregularities may have been due either to differences in the patients' sera, or to differences in the complements used. Patients' sera may differ in sensitising power; and complements may differ in rapidity of action, in capacity for being adsorbed, and in capacity for destroying the anticomplementary power of the antigen-antibody complex. The influence of these idiosyncrasies upon complement destruction would not be identical at each of the three temperatures employed.

Otto Müller (1915)* investigated the influence of temperature on specific fixation of the complement, using horse, human, and sheep sera and the corresponding antisera prepared from rabbits.

He found that, with sufficiently long exposure, specific complement fixation was generally better in the cold (finely broken ice, giving a temperature of 0° C.) than in the warm (incubator). In the cold a smaller amount of antigen sufficed to

* *Zeitschr. f. Immunitätsforschung*, Orig. XXIII, p. 306.

give an equal degree of complement fixation. Given small but sufficient doses of antigen, the dose of antiserum needed for complement fixation was smaller in the cold than in the warm; with larger doses of antigen, it happened sometimes, but not invariably, that fixation was better in the warm, apparently because the optimum for cold fixation had been exceeded.

When antigen, antiserum, and complement were digested for only a short time, warm fixation was stronger, but on longer digestion there was a clear balance in favour of cold fixation. This difference he attributed to (1) the favourable influence of warmth on the antigen-antibody reaction (first phase of complement fixation) and (2) the favourable influence of cold on the second phase of the reaction (anticomplementary action).

Variations in experimental results could thus be explained by recognising that the demonstrable degree of complement fixation was the resultant of two reactions which were divergently influenced by differences in temperature. Consistently with this explanation, it was often found that, after preliminary digestion of antigen and antiserum, a short period of complement fixation was sufficient to give better results by the cold method.

In the experiments which Müller recorded in detail, his usual time of exposure to each temperature was 1—1¼ hours. In certain experiments, comparing different times at each temperature, his times were 10 minutes, 30 minutes, 1 hour, 1½ hours, 2 hours. The optimum for cold fixation was not always reached in 1½ hours, results at 2 hours being rather stronger.

On the assumption that chemical activity plays an important part in the reaction at 37° C., the following comments may be made on Müller's observations :—

(1) Fixation is (*a*) generally better in the cold, but (*b*) not so rapid. This may be because (*a*) at 37° C. the anticomplementary power of the antigen-antibody complex is destroyed before so much complement is fixed, but (*b*) at 0° C. complement is fixed more by adsorption and less by chemical action, and adsorption is a relatively slow process.

(2) In the cold, less antigen is needed. As above, a given amount of complement, acting more rapidly at 37° C., may destroy all the anticomplementary power of a relatively small amount of sensitised antigen before all the complement is used up; but, when the same quantities are treated at 0° C., the process is slower and all the complement is exhausted when the end-point is reached.

(3) In the cold, less antiserum is needed. With relatively weak sensitisation, there may be less loss of complement by chemical interaction at 37° C.

(4) With large doses of antigen, fixation was sometimes better at 37° C. Assuming that the end-point at 0° C. had been reached, though it is quite probable that this was not the case, the large amount of sensitised antigen would involve large loss of complement by chemical action at 37° C.; and this might not be accompanied by corresponding increase in the adsorptive capacity of the antigen-antibody complex at 0° C.

(5) With short exposure, warm fixation was stronger; but with longer exposure cold fixation was stronger. As suggested above, the two principles in operation may be chemical action (greater at 37° C. but rapidly ceasing) and adsorption (stronger at 0° C. and more prolonged though slower).

(6) After preliminary incubation of antigen and antiserum, strong complement fixation was rapidly attained in the cold. The preliminary incubation may have accelerated the formation of an adsorbing antigen-antibody complex.

Mundt (1915)* found that the capacity of organ emulsions to absorb the Wassermann substance from syphilitic sera was not lost by repeated boiling of the emulsion at 100° C.; it was, however, removed, by repeated extraction with alcohol, though one or two extractions might not be enough.

The greater part of the absorption took place at the moment of contact. When an emulsion of human heart was used for absorption, the concentration of the serum had no essential influence on the result of the reaction in the warm, but in the cold increased dilution yielded less absorption. Accordingly, when the warm and the cold method were compared with diluted serum, warm absorption was usually somewhat stronger, but with concentrated serum cold absorption was the more potent.

Mundt absorbed inactivated serum with human heart extract by both the cold and the warm methods, and then estimated the amount of absorbed Wassermann substance by the ordinary Wassermann test (warm method), and also by the cold fixation method of Jacobsthal. He found that, after absorption in the warm, the serum fixed complement better in the cold, but after absorption in the cold it fixed complement better in the warm. Hence he suggested, hypothetically, that in positive sera there were two reacting bodies, and that, for the fixation of these by extract, cold was better for the one and warm for the other. The fact that liver emulsion did not bring out these points so clearly as heart emulsion helped to explain why Jacobsthal's cold fixation method gave better results with heart than with liver extract.

The artificial "Wassermann bodies," obtained by digesting normal serum with bacteria or agar, were not absorbed by organ cells; they were thus essentially different from the true syphilitic bodies.

In his cold fixation experiments, Mundt kept the mixture of extract, serum, and complement for one hour in the ice-chest. His absorption experiments with organ cells were usually for times ranging up to one hour, both at 37° C. (water-bath) and at 0° C.

It is probable that Mundt (like the preceding investigators) did not give the cold method sufficient time to obtain maximum adsorption of "Wassermann substance" by antigen. Had he done so, his results might possibly have been compatible with the simple explanation that cold adsorption proceeds more slowly, but, given adequate time, is more effective in picking out the Wassermann substance from a weak serum.

It might also be suggested that his organ emulsions acted in two ways. (1) In virtue of the alcohol-soluble substances which they contained, they took up (like a chemical solvent) the "Wassermann substance" contained in the serum; and (2) in virtue of their colloidal properties, they adsorbed

* *Zeitschr. f. Immunitätsforschung.*, Orig. XXII., p. 267.

the constituents of the serum with which "Wassermann substance" was bound. (1) might be more potent at 37° C. and relatively little affected by dilution of the serum; and (2) might be more efficient at 0° C., but might be readily weakened by dilution.

After treatment with organ emulsions at 37° C., the supernatant serum fixed complement better by the cold method. This may have been because treatment at 37° C. involved more of process (1)—chemical extraction—than of process (2)—removal of adsorbable elements; and hence the residual serum acted better under the condition (0° C.) where the presence of these remaining adsorbable elements would create a strong anticomplementary complex.

For the converse reason, after treatment at 0° C. there would be left in the serum more material capable of uniting with the Wassermann antigen under process (1), which would be favoured by performing the W.R. at 37° C.

It is interesting to note that, in Mundt's experiments, sera artificially made "positive" did not behave like syphilitic sera.

H. Sachs and K. Altmann (1917)* investigated the influence of temperature and the reaction of the medium in the Wassermann test.

In a neutral medium the majority of sera reacted alike with fixation at 0° C. and fixation at 37° C., though some differed, giving a positive at 0° and a negative at 37°, or *vice versâ*. In the course of their work they observed that, amongst the sera which agreed at both temperatures when tested in the usual way, differences could sometimes be brought out when the tests were made with diminished amounts of serum; they therefore found it useful to dilute their sera 1 : 20 instead of employing the more usual 1 : 10.

Fixation in the presence of graduated quantities of acid produced interesting results, which were illustrated in the following experiment:—

To each quantity of HCl, made up to 0·5 c.c., was added 0·5 c.c. of 1 : 20 inactivated patient's serum, together with 0·25 c.c. of 1 : 6 alcoholic organ extract and 0·25 c.c. of 1 : 5 complement. One series was incubated at 37° C. for $1\frac{1}{4}$ hours, and a second series was kept at 0° C. for the same length of time. Then 1 c.c. of corpuscles and amboceptor was added, and the tubes were incubated. The results were:—

Amount of HCl.	Resultant Hæmolysis after	
	fixation at 37° C.	fixation at 0° C.
$\frac{1}{400}$ 0·5 c.c.	o	k
0·4 c.c.	o	k
0·3 c.c.	Spch.	k
$\frac{1}{800}$ 0·5 c.c.	f.k.	k
0·4 c.c.	k	k
0·3 c.c.	k	st.
0·25 c.c.	k	Sp.
0·2 c.c.	k	Spch.
0	k	o

o = none; Spch. = slight trace; Sp. = trace; st. = marked; f.k. = almost complete; k = complete.

* *Zeitschr. f. Immunitätsforschung*, Orig. XXVI., p. 460.

This serum, as shown in the tubes without HCl, was one which, in a neutral medium, was positive at 0° C. and negative at 37° C.

They thus showed that, with the aid of HCl, they could make this same serum give the following results:—

(1) No HCl; + at 0° C., − at 37° C.
(2) + 0·4 of $\frac{1}{800}$ HCl; − „ − „
(3) + 0·4 of $\frac{1}{400}$ HCl; − „ + „

And with other sera, which were positive at both 0° C. and 37° C., they found that the + reaction was removed at the lower temperature with relatively small quantities of HCl, but much more acid was required to remove it at 37° C. Thus their general conclusion was that HCl could annul a positive Wassermann reaction more easily with cold than with warm fixation.

In explanation of these results, they attached importance to Friedemann's view that complement fixation depended on the anticomplementary action of the serum globulins and considered that this action was more powerful in the cold than at 37° C. This action was also promoted by HCl, and hence a serum, negative at 37° C. in a neutral medium, could be made positive by the assistance of this reagent, in suitable strength. There was, however, an optimum strength, excess of which was inhibitory; and this optimum was much lower with cold than with warm fixation.

These results were obtained with inactivated sera and, as inactivation tended to move a serum in the alkaline direction, they were compatible with the observations of Altmann and Zimmern, who had found that active sera always reacted more strongly with warm than with cold fixation; but, after inactivation, some of them reacted better by the warm method and others by the cold treatment. Movement away from the acid direction would bring into prominence the above-mentioned difference between the optimum effect of acid when applied to warm and to cold fixation, a difference which, Sachs and Altmann thought, might perhaps be attributable to "secondary" properties of the serum.

On comparing and contrasting the influence of the addition of NaOH with the action of HCl, the authors failed to obtain uniform results, but did not obtain evidence which might invalidate the conclusions which they based on the action of HCl.

It is known that slight difference in the acidity of the medium may modify a colloidal reaction, and it is probable that this influence is not quite the same at 0° C. as at 37° C., particularly when complement is one of the reagents. The experiments of Sachs and Altmann appear to illustrate these points, but it is not clear that they prove anything further.

The Present Position.

In tests for admittedly specific antigen-antibody reactions, cold fixation does not appear, so far, to have been tried on a

large scale ; therefore there is not sufficient evidence to enable one to form an opinion as to its practical value for such tests. On the theoretical side, the data which I have quoted are of interest. Exposure to low temperature places an artificial restraint upon the activity of complement and results, it would appear, in enhancing the anticomplementary properties of the specific antigen-antibody complex. The explanation suggested is that complement is fixed by adsorption ; adsorption is best obtained by promoting the slow formation of a finely divided precipitate in the interaction between antigen and antiserum ; and these conditions are favoured by allowing the interaction to take place at a low temperature.

This is an important theory, but it does not appear to have received general acceptance, because the majority of investigators continue to employ the warm method in specific complement fixation work. It seems to me that it rests with these investigators to formulate their objections to the cold method. Controversy on this subject would probably be the best way of clarifying knowledge about the mechanism of complement fixation.

It is not clear whether this mechanism is the same in the W.R. as in admittedly specific antigen-antibody reactions. The laboratory data which I have quoted may, perhaps, be taken as an indication that here also cold fixation enhances anticomplementary action in a way which increases the delicacy of the test without impairing its reliability. I must refer to the report by Drs. Griffith and Scott (pp. 7–77) for further investigations on this subject.

Controversial Issues about Wassermann Substance.

I concluded Part I. of this report with the observation that more controversy is required about the main issues as to the nature of complement before one can attempt to formulate any general conclusions. The same remark applies to Wassermann substance. At the same time one cannot feel satisfied with the statement, to be found in most text-books, that the true nature of the Wassermann reaction is quite unknown. A good many important facts about it are known. At present they cannot all be focussed under one theory, but they are valuable contributions to rival theories and should serve to stimulate controversy about the main issues which must be fought out before the test can be satisfactorily explained.

Put crudely, the main issue lies between the biological conception and the physical conception. The following is one way of expressing the former view.

If the Wassermann test is really based on a biological process peculiar to syphilis, in its performance there should be

reproduced some feature characteristic of the metabolism in the plasma* of the syphilitic subject.

The plasma of the syphilitic differs from that of the normal person in two respects: (1) Owing to the action of the spirochaetes, there is tissue destruction, with probably a breaking down of protein-lipoid combinations, and some of this material is constantly entering the circulation, where it has to be assimilated. (2) During the course of the disease the plasma is modified (or becomes capable of modifying the tissues) in a special manner. This modification, according to one view, may be regarded as the production of a new substance ("antibody") which helps the plasma ("complement") to assimilate the disintegration products ("antigen") produced by the spirochaetes. As the new substance increases when the disease becomes more active, decreases in quiescent periods, and disappears when a cure is effected, its existence seems to be in close dependence on the supply of circulating "antigen." The new substance may be a newly created reaction-product or possibly it may be nothing more than the modified residue of circulating "antigen," after the latter has been digested, so far as it can be digested, by the constituents of the plasma. On either view, the "antigen" of the syphilitic is not like ordinary antigens (*e.g.*, bacteria, or alien red corpuscles), because, though abnormal, it is derived from native tissue products; and, on the latter view, the term "antibody" is also used in a special sense, because, though it is the function of syphilitic "antibody" to sensitise "antigen," this "antibody" is not a newly created antagonistic substance, but simply a residual product of "antigen."

If the above ideas are applicable to the principles underlying the Wassermann test, the artificial antigen might represent the undigested lipoidal disintegration products freshly turned out into the plasma; these would combine with the residue of semi-digested lipoidal compounds or reaction-products contained in the syphilitic serum. In virtue of this combination, the two substances would be rendered amenable to the action of complement, the activity of which would be used up in the reaction.

There are, of course, several other ways of expressing what may be termed a biological conception of the reaction. One might, for example, substitute Meinicke's idea (pp. 188–9). According to him, the essential character of syphilitic serum is the presence of new chemical constituents, peculiar to syphilitics, which are bound in a relatively firm complex with the antigen lipoids; the precipitability of syphilitic sera is an accessory factor which does not express the essential nature of the reaction. And Gärtner's views (p. 192) are another way of expressing the idea that the W.R. is a reaction with specific "Wassermann substance," irrespective of the precipitability of syphilitic serum.

* With reference to the functions of the plasma, *see* footnote on p. 83.

Views such as the above are all definitely at variance with the physical conception which maintains, in one form or another, that the W.R. has no essential feature in common with specific immunity reactions.

This conflict of opinion should be a useful stimulus to controversy upon a definite issue.

Several ways of raising this issue are evident from the data collected in this report.

What is the significance of the fact that syphilitic sera are more readily precipitated than normal sera? Is this a diagnostic feature of syphilitic serum or only a secondary and more or less unreliable characteristic? Is the W.R. essentially a precipitation test, in which complement is merely used as an indicator to show that precipitation has occurred? If so, is it justifiable to replace the W.R. by a test, such as that recommended by Sachs and Georgi, which demonstrates precipitation without the use of complement?

Then there is the work on "artificial positives." Do the reactions obtained with these throw any light on the nature of syphilitic sera?

It is still far from settled to what extent, if any, the W.R. is really analogous to a reaction between admittedly specific antigen and antibody. In such specific tests, what are the relations between complement and sensitised antigen, and how do these relations compare with the interaction between complement, "antigen," and syphilitic serum? Pursuit of these questions of course involves, and may help to clear up, unsettled problems about the nature of complement.

The importance of lipoids in the W.R. raises wider questions as to the function of these bodies, or of lipoid-protein combinations, in the production of true antibodies. Do they produce specific antibodies by acting upon the lipoidal constituents of cell-membranes in the animal undergoing immunisation?

Then one comes to still wider questions as to the meaning of specificity. How is this term to be defined by those who consider that the characteristics of syphilitic serum are due to a specific disturbance of the physical properties of the serum? Or, if a special antibody is present, how is it formed and in what sense is it specific? What are the relations of an antibody to the chemico-physical constitution of its antigen?

The controversial issues raised by questions such as the above are not the same as the old issues which have been fought out by Ehrlich and Bordet. In particular, the importance of recognising colloidal principles is no longer in dispute. Principles such as the following will, I think, be accepted as exercising an important influence upon immunity reactions:—

(1) The surfaces of colloidal particles adsorb certain protective substances, *e.g.*, the substances termed by Herzfeld and Klinger "break-up products," which keep

the particles in suspension and prevent them from coalescing.

(2) When these protective substances are diminished, there is a tendency for the particles to coalesce, and this aggregation of the particles may lead to the formation of a precipitate. This is particularly the case with the larger particles termed globulins.

(3) On the other hand, colloidal reactions may equally well bring about increased adsorption of protective substances, thus promoting dissemination instead of clumping.

(4) The precipitate formed in an antigen-antibody reaction adsorbs complement, and this adsorption may take place when the precipitate is too fine to be visible to the naked eye.

(5) All the above reactions depend on the particular chemical characters of the colloids and of the substances which are attracted to them, as well as on the chemico-physical conditions of the medium in which the reaction takes place.

(6) The infinite variety of antigens is quite compatible with the infinite variety of colloidal conditions.

The above are suggested as a few examples of colloidal principles which appear to be accepted in immunity work. They are only given as instances of the undoubted fact that these principles serve partly to explain the mechanism of immunity reactions. They are obviously not the whole of the explanation, because, as is evident from the work of the biochemists, the chemical interactions which are involved are equally important, though they are at present very imperfectly understood.

Taking the importance of colloidal principles for granted, controversial issues must turn upon that unexplained part of the mechanism of immunity reactions which should account for specificity.

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